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The endocannabinoid system: a key modulator of stress effects on memory

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THE ENDOCANNABINOID SYSTEM: A KEY MODULATOR OF STRESS EFFECTS ON MEMORY

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GENERAL INTRODUCTION

The endocannabinoid system

The discovery of the main psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC) led to the identification of the endogenous cannabinoid system (Gaoni and Mechoulam, 1964). The main constituents of the endocannabinoid system consist in the cannabinoid receptor type 1 and type 2 (CB1 and CB2, respectively) (Devane *et al*, 1992; Herkenham *et al*, 1990; Matsuda *et al*, 1990) and the two major endogenous ligands for these receptors, the *N*-arachidonoyl ethanolamine (anandamide, AEA) (Devane *et al*, 1992) and the 2-arachidonoyl glycerol (2-AG) (Sugiura *et al*, 1995). Endocannabinoids are synthesized on demand from phospholipid precursors on the postsynaptic membrane by Ca^{2+}-dependent and independent mechanisms (Kano *et al*, 2009). This lipophilic molecules are released directly into the synaptic cleft and act in retrograde fashion on the presynaptic neuron where the cannabinoid receptors are expressed. Activation of the CB1 receptor modulates intracellular transduction pathways through activation/inhibition of several ion channels and kinases, thus inducing the inhibition of further neurotransmitter release (Kano *et al*, 2009; Turu and Hunyady, 2010). AEA and 2-AG are subsequently taken back into the cell by a still poorly defined uptake process mediated by a transporter mechanism (Fu *et al*, 2011; Hillard *et al*, 1997) and enzymatically degraded by the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Kano *et al*, 2009), respectively.

CB receptors couple to $G_{i/o}$ proteins which function to inhibit adenylyl cyclase activity, activate potassium channels and inhibit voltage-gated calcium channels (Howlett *et al*, 2002). CB1 receptors represent the most abundant class of G-protein-coupled receptors in the central nervous system, but are also present in a variety of peripheral tissues (Howlett *et al*, 2002), while CB2 receptors are mostly peripherally located on immunological tissues and, only recently, immunohistochemical analyses have revealed the presence of CB2 receptors in neuronal and glial cells in diverse rat brain areas, including the cerebellum and hippocampus (Onaivi *et al*, 2006; Van Sickle *et al*, 2005). Within the limbic system, the most prominent expression of the CB1 receptor can be seen in the hippocampus, the basolateral complex of the amygdala (BLA), and prefrontal cortex (PFC) (Marsicano and Kuner, 2008; McPartland *et al*, 2007). Only recently, CB1 mRNA expression has been clearly detected at low levels in the central nucleus of the amygdala (CeA) (Kamprath *et al*, 2010). Similar to the CB1 receptor,

FAAH and MAGL are found in high levels in the BLA, whereas only low levels can be found in the CeA (Ramikie and Patel, 2012). Within these limbic regions, CB1 receptor is expressed at very high levels in cholecystokinin-positive GABAergic interneurons (Azad *et al*, 2008; Marsicano and Lutz, 1999; Morozov *et al*, 2009) and at moderate to low levels in glutamatergic terminals (Kano *et al*, 2009; Kawamura *et al*, 2006; Monory *et al*, 2006). However, this receptor has also been detected on serotonergic, noradrenergic and dopaminergic terminals (Haring *et al*, 2007; Hermann *et al*, 2002; Oropeza *et al*, 2007). CB1 receptors located on GABAergic axon terminals are activated by lower concentrations of cannabinoid receptor agonists than CB1 receptors located on glutamatergic terminals (Hoffman *et al*, 2007; Ohno-Shosaku *et al*, 2001). Endocannabinoids and CB1 receptors have been shown to differentially mediate homeostatic, short- and long-term synaptic plasticity processes throughout the brain (Chevaleyre *et al*, 2006; Katona and Freund, 2012; Marsicano and Lutz, 2006), thus the cannabinoid system has been reported to be crucially involved in learning and memory processes (Akirav, 2011; Campolongo *et al*, 2009; Kano *et al*, 2009; Marsicano and Lafenetre, 2009; Wotjak, 2005).

Role of the endocannabinoid system in the modulation of emotional memory

Emotional learning is extremely important for the survival of an individual; indeed emotionally arousing events typically leave lasting and vivid memories (McGaugh, 2000). The most prominent consequence of a stressful experience is a quick release of epinephrine from the adrenal medulla that allows the organism to liberate energy sources for immediate action. Consequently norepinephrine is released in the brain via the stimulation of the vagal nerve and the nucleus tractus solitarius (NTS). As part of the response, neurons, including principal cells in the amygdala, receive a strong noradrenergic input from the locus coeruleus (LC) shortly after stress (Sara, 2009). The same neurons also receive high levels of the adrenocortical hormone corticosterone (CORT, in rodents; cortisol in humans) which binds with higher affinity to mineralcorticoid receptors (MRs) and lower affinity to glucocorticoid receptors (GRs) (Reul and de Kloet, 1985). During stress conditions MRs and GRs are occupied and mediate both genomic and rapid non-genomic actions (Joels and Baram, 2009; Tasker *et al*, 2006). Although noradrenaline and CORT are key elements in the stress response, they accomplish their full effect in concert with several other transmitters, thus mediating the effect of stress and emotional arousal on memory (Joels *et al*, 2011; McGaugh and

Roozendaal, 2002). In this scenario the endocannabinoid system has emerged as a key modulator of stress effects on memory function. Compelling evidence has been reported in the literature demonstrating a strong bidirectional interaction between endocannabinoids and stress-activated hormones such as glucocorticoids and norepinephrine. For instance it has been demonstrated that, in the central nervous system, glucocorticoids induce endocannabinoid signaling in the hypothalamus (Di *et al*, 2003). Endocannabinoids, then, act retrogradely to inhibit the release of glutamate in the paraventricular nucleus and suppress HPA axis activity (Di *et al*, 2003; Di *et al*, 2005). Conversely, both stress and glucocorticoids significantly alter endocannabinoid content in limbic brain regions resulting in opposing actions that can both increase and terminate the stress response (Hill and McEwen, 2010a). Furthermore the endocannabinoid system mediates stress responses and emotional homeostasis, in part, by targeting noradrenergic circuits (Morrish *et al*, 2009). Early anatomical studies have identified moderate CB1 binding and CB1 mRNA in the principal noradrenergic nuclei, the LC and the NTS (Derbenev *et al*, 2004; Herkenham *et al*, 1991; Jelsing *et al*, 2008; Mailleux and Vanderhaeghen, 1992; Matsuda *et al*, 1993). The existence of CB1 receptors in the LC and NTS suggests that cannabinoids may modulate noradrenergic activity. Cannabinoids have been shown to increase noradrenergic release in the PFC, (Oropeza *et al*, 2005). Moreover Muntoni et al. (2006) demonstrated that intravenous injection of the cannabinoid agonists WIN55,212-2 or Δ^9-THC, dose-dependently increases the firing rate of LC noradrenergic neurons *via* the activation of CB1 receptors (Muntoni *et al*, 2006). Given the evidence that CB1 receptors were found to be abundantly expressed within the limbic system and to crucially modulate synaptic transmission (Katona *et al*, 2001; Tan *et al*, 2011) and neuronal firing (Pistis *et al*, 2004), the cannabinoid system has been increasingly emerged as an important modulator of different learning and memory processes (Akirav, 2011; Campolongo *et al*, 2009; Kano *et al*, 2009; Marsicano *et al*, 2009; Wotjak, 2005).

The brain regions mainly involved in emotionality are represented by subcortical limbic structures, such as the amygdala, hippocampus, ventral striatum, and thalamus, as well as cortical structures, including the anterior cingulate cortex and medial and orbital regions of the PFC (Price and Drevets, 2010). In this assembly, the amygdala represents a key region for the association between environmental information with emotional significance for fear and anxiety responses but also for processing of positive emotions (Aggleton, 1993;

Baxter and Murray, 2002; Davis *et al*, 1994; Pape and Pare, 2010). In particular, considerable evidence indicates that emotional memory modulation requires activation of the BLA specifically. Lesions of the BLA, but not the CeA, block the memory enhancing effects of systemic GR activation on inhibitory avoidance retention (Roozendaal and McGaugh, 1996). During emotionally arousing training norepinephrine is also released into the amygdala (Galvez *et al*, 1996; McIntyre *et al*, 2002; Quirarte *et al*, 1998), whereas attenuation of noradrenergic signaling with the β-adrenoceptor antagonists propranolol or atenolol infused into the BLA, but not into the CeA, blocked the memory enhancement induced by a glucocorticoid administered either systemically or directly into the BLA (Quirarte *et al*, 1997; Roozendaal *et al*, 2002). Considerable evidence indicates that glucocorticoids interact with this training-associated noradrenergic activation within the amygdala in enhancing the consolidation of memory of emotionally arousing training experiences (Roozendaal *et al*, 2009). In concert with this glucocorticoid-noradrenergic interaction, it has been recently shown that the endocannabinoid system plays a crucial role in modulating neural processes underlying emotional memory consolidation. It has been reported that the cannabinoid receptor antagonist AM251 blocks the ability of systemically injected corticosterone (or the synthetic analogue dexamethasone) to enhance memory consolidation of an inhibitory avoidance training when directly infused into the BLA (Campolongo *et al*, 2009) or into the hippocampus (de Oliveira Alvares *et al*, 2010). These findings provided the first *in vivo* evidence in mammals of the existence of this pathway (Hill *et al*, 2010b). Besides the enhancing effects of glucocorticoids on memory consolidation, many studies demonstrated that such hormones typically impairs memory retrieval and working memory during emotionally arousing test situations (de Quervain *et al*, 2009; de Quervain *et al*, 1998; Roozendaal, 2000; Roozendaal *et al*, 2004). Recently, the interaction between glucocorticoids and the endocannabinoid system in modulating contextual fear memory retrieval has been examined. The cannabinoid antagonist AM251 infused into the dorsal hippocampus blocked the impairing effects on memory retrieval of systemic administered corticosterone; such impairing effects were mediated by elevation of hippocampal 2-AG. Moreover, the β-adrenoceptor antagonist propranolol blocked the impairing effect of WIN55,212-2 on memory retrieval and, conversely, the CB1 receptor antagonist AM251 infused into hippocampus together with an impairing dose of norepinephrine failed to abolish the impairing effect of norepinephrine on memory retrieval (Atsak *et al*, 2012).

Collectively, these findings indicate that endocannabinoids interact with glucocorticoids and, depending on the availability of arousal-induced activation of noradrenergic system, they might differentially modulate memory functions. However, despite quite few studies in the literature, there is no consensus regarding the direction of endocannabinoid effects on memory; conflicting effects of cannabinoid compounds have been reported in many different behavioral paradigms and on different memory processes.

Early studies, examining the effects of systemic pretraining administration of the cannabinoid agonists Δ^9-THC and WIN55,212-2 on memory acquisition reported impairing effects on several behavioral tasks in rodents (Da and Takahashi, 2002; Lichtman *et al*, 1995; Pamplona and Takahashi, 2006b), whereas other studies reported that intraperitoneal administration of the cannabinoid antagonist rimonabant induces similar effects to those induced by the agonists by impairing spatial memory acquisition (Robinson *et al*, 2008). Similarly, indirect cannabinoid receptor agonism impairs memory acquisition in a recognition memory task (Campolongo *et al*, 2012) and in the inhibitory avoidance task (Mazzola *et al*, 2009). Local infusions into distinct brain regions have illustrated more clear results in this regard. Pretraining administration of a CB1 receptor agonist into the hippocampus has consistently been shown to impair spatial learning (Abush and Akirav, 2010; Egashira *et al*, 2002; Lichtman *et al*, 1995; Wegener *et al*, 2008), whereas bilateral blockade of BLA CB1 receptor transmission has been reported to prevent the acquisition of associative fear memory in an olfactory fear conditioning paradigm (Tan *et al*, 2011).

Conflicting data have been also reported concerning cannabinoid effect on memory consolidation. Systemic posttraining administration of the FAAH inhibitor URB597, which increases AEA levels only in those brain regions where it is endogenously released, has been shown to impair memory consolidation in an object recognition task (Busquets-Garcia *et al*, 2011). Posttraining intra-hippocampal administration of the synthetic cannabinoid receptor agonist WIN55,212-2 has been reported to impair memory consolidation (Jamali-Raeufy *et al*, 2011; Yim *et al*, 2008; Zarrindast *et al*, 2011). However, other authors reported enhancing effects of AEA in the hippocampus (De Oliveira Alvares *et al*, 2008) and of WIN55,212-2 in the BLA (Campolongo *et al*, 2009).

Cannabinoid effects on memory retrieval have been often reported to be detrimental when administered either systemically (Mishima *et al*, 2001; Niyuhire

et al, 2007) or in discrete brain areas (Atsak *et al*, 2012; Piri and Zarrindast, 2011; Segev and Akirav, 2011).

There is a general agreement concerning cannabinoid effects on memory extinction where cannabinoids seem to modulate the facilitation of this memory process. Using fear conditioning procedure, Marsicano et al. (2002) and subsequent investigators demonstrated that inhibition of endocannabinoid transmission robustly inhibits fear extinction (Abush *et al*, 2010; Marsicano *et al*, 2002; Pamplona *et al*, 2006a; Suzuki *et al*, 2004). Conversely, stimulation of endocannabinoid transmission accelerates fear extinction (Barad *et al*, 2006; Chhatwal *et al*, 2005; Suzuki *et al*, 2004).

Although such discrepant findings, collectively, the endocannabinoid system appears to be strongly involved in different memory processes by influencing multiple brain regions. Differences in handling procedures, experimental conditions, behavioral tasks, doses, and the drug administered may account for the diversity of findings reported. However, variations in the stressful conditions employed in the different studies are implicated as well. The neural processes underlying emotional memory formation seems to be differently sensitive to cannabinoids depending on the levels of emotional arousal associated to the experimental context (see Chapter 5).

Outline

As mentioned above, a large amount of evidence indicates that the endocannabinoid system is crucially involved in the modulation of memory consolidation for stressful experiences (Akirav, 2011; Campolongo *et al*, 2009; Kano *et al*, 2009; Marsicano *et al*, 2009; Wotjak, 2005). Indeed previous findings from our laboratory have demonstrated that CB1 receptor activation within the BLA enhances memory consolidation. In particular, the cannabinoid agonist WIN55,212-2, bilaterally infused into the BLA immediately after inhibitory avoidance training, enhanced memory consolidation. Conversely, the CB1 receptor antagonist AM251 administered after training into the BLA dose-dependently impaired 48-h inhibitory avoidance retention (Campolongo *et al*, 2009). Based on these previous findings we hypothesized that after an aversive experience endocannabinoids might be released within the BLA in order to modulate the better storage of emotionally salient events.

Chapter 1 explores more in deep the driven hypothesis by investigating whether, after experiencing a traumatic event, endocannabinoids are released within the BLA and other limbic structures, such as the hippocampus and the medial PFC. Further, this chapter examines the effects induced by exogenous augmentation of the endocannabinoid system in the same brain regions on memory for emotionally salient events. Given the well-established central role of the BLA in mediating emotional memory consolidation (McGaugh, 2000), Chapter 2 also investigates the role of endocannabinoid system within the BLA in modulating the functional interactions with the hippocampus and mPFC during aversive memory consolidation.

In 2003, Patel et al. demonstrated that the intravenous anesthetic agent propofol is able to increase AEA brain levels in mice through the inhibition of FAAH (Patel *et al*, 2003). Clinical data have demonstrated that the use of propofol immediately after experiencing a traumatic event (i.e. car accidents, myocardial infarctions) and during intensive care unit treatment (ICU) is associated with a higher incidence of traumatic memories from perioperative awareness and ICU treatment as compared to other general anesthetics (Jones *et al*, 2007). These effects could result in stress-related disorders such as posttraumatic stress disorder and impaired long-term health-related quality of life outcomes (Kapfhammer *et al*, 2004; Schelling *et al*, 2003). This evidence together with our preliminary results showing that endocannabinoids are released after an aversive experience in order to modulate the better storage of emotionally salient events, we investigated (**Chapter 2**) whether propofol administration would modulate aversive memory retention of an inhibitory avoidance task, and whether any possible effect could be mediated by the indirect activation of the endocannabinoid system in rats.

The complexity of endocannabinoid neuromodulation and the diversity of task-dependent activation of neuronal circuits makes endocannabinoid effects on behavior being strongly dependent on environmental conditions (Zanettini *et al*, 2011). It is well established that cannabinoid compounds induce biphasic effects on emotionality both in humans (Del Porto and Masur, 1984; Reilly *et al*, 1998; Velez and Ungemack, 1989; Zinberg, 1984) and in rodents (Campos *et al*, 2010; Haller *et al*, 2009; Sciolino *et al*, 2011; Zanettini *et al*, 2011). As described above, pharmacological manipulation of the endocannabinoid system has been described to induce contradictory effects on memory function. **Chapter 3** explores the

possibility that cannabinoid effects on memory could be strongly dependent upon the arousal state of the animal at the time of testing. In particular, we investigated the interaction between cannabinoids and glucocorticoids in the modulation of both short- and long-term recognition memory in rats under two experimental conditions that differed in the level of novelty-induced emotional arousal at the time of encoding.

Likewise, it has been shown that variation in environmental aversiveness differentially influences spatial memory retrieval in rats (Akirav *et al*, 2004; Salehi *et al*, 2010) and, as described above, that glucocorticoids interact with the hippocampal endocannabinoid system in impairing aversive memory retrieval in a contextual fear conditioning paradigm (Atsak *et al*, 2012). Based on these findings, **Chapter 4** investigates whether the level of stress associated to the experimental context could influence endocannabinoid modulation of spatial memory retrieval in rats.

Chapter 5 provides an overview of the existing literature regarding the cannabinoid effects on different memory phases, focusing on the interaction between the endocannabinoid system and the level of stress associated to the experimental context or arising from previous aversive experiences. This chapter finalizes providing possible assumptions to explain the opposing effects of cannabinoid on cognitive processes reported in the literature.

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CHAPTER 1

ENDOCANNABINOIDS ENHANCE MEMORY CONSOLIDATION OF EMOTIONALLY AROUSING EXPERIENCES: KEY ROLE OF THE BASOLATERAL COMPLEX OF THE AMYGDALA

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In preparation

Abstract

Extensive evidence indicates that the basolateral complex of the amygdala (BLA), hippocampus and medial prefrontal cortex (mPFC) modulate the consolidation of memory for emotionally arousing experiences. In this study, we first investigated to what extent the level of emotional arousal at the time of training would influence 48-h memory retention of an inhibitory avoidance task. To this aim three groups of rats were trained under different inhibitory avoidance experimental conditions (no, low, high footshock) and tested for memory retention 48-h later. Rats trained under the high footshock (0.45 mA) condition presented an enhancement of 48-h memory retention. We next investigated the role of the endocannabinoid system in the amygdala, hippocampus and mPFC in memory consolidation. To examine whether endocannabinoids are normally released after arousing inhibitory avoidance training, we measured anandamide (AEA) and 2-arachidonoylglycerol (2-AG) endocannabinoid levels in these three brain regions of rats 10, 30 or 60 min after training on the different experimental conditions considered. We found that rats trained with the high footshock had higher levels of AEA in the amygdala, hippocampus and mPFC than rats that were only exposed to the inhibitory avoidance apparatus but not shocked. We therefore tested whether the AEA hydrolysis inhibitor URB597, which elevates AEA levels, might enhance memory consolidation of this training. Immediate posttraining infusions of URB597 into the BLA, hippocampus and mPFC enhanced 48-h inhibitory avoidance retention performance. Further, separate groups of rats were co-administered with URB597 together with a nonimpairing dose of the CB1 receptor antagonist AM251 in the three brain regions considered. We found that URB597 memory enhancing effects were blocked by the concomitant infusion of AM251, thus demonstrating the involvement of CB1 receptors in such memory effects. In the last set of experiments, we investigated the role of the BLA in modulating the endocannabinoid response in other brain regions. We measured endocannabinoid levels in the hippocampus and mPFC or infused URB597 into these two brain regions after inhibitory avoidance training of rats with bilateral permanent lesions of the BLA. We found that there was neither an increase of endocannabinoid levels nor an URB597-induced enhancement of memory consolidation in the lesioned rats. Our findings provide evidence that the endocannabinoid system enhances the consolidation of memory of emotionally arousing inhibitory avoidance training and, interestingly, that the BLA plays a crucial role in modulating the endocannabinoid response during and after aversive experiences.

Introduction

Emotionality plays a key role in learning and memory processes and stressful experiences tend to leave lasting and vivid memories (McGaugh, 2000). The neurocircuitry controlling emotional behavior is represented by the limbic system; it consists of several brain regions functionally interconnected, such as the amygdala, the hippocampus, the medial prefrontal cortex (mPFC), the ventral striatum, the hypothalamus, the medial thalamus (Price and Drevets, 2010). An appropriate emotional response to an aversive event requires fine-tuned neurotransmitter release regulation as well as functional neuronal circuits (Gold, 2004; McEwen, 2012; McGaugh, 2000). In the last decades the endogenous cannabinoid system has is emerged as a possible key modulator of such functions. It consists of the G-protein coupled cannabinoid 1 and 2 receptors (CB1/2), their endogenous ligands so called endocannabinoids, and synthetic and metabolizing enzymes (Devane *et al*, 1988; Herkenham *et al*, 1991; Herkenham *et al*, 1990). Endocannabinoids are lipidic molecules not stored in vesicles but synthesized on demand and released from postsynaptic neurons into the synaptic cleft. They travel retrogradely and bind cannabinoid receptors presynaptically located (Kano *et al*, 2009). The most characterized endocannbainoids are *N*-arachidonoyl ethanolamine (anandamide, AEA) (Devane *et al*, 1992) and 2-arachidonoyl glycerol (2-AG) (Sugiura *et al*, 1995). AEA and 2-AG signaling is mainly deactivated by the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL), respectively (Kano *et al*, 2009). CB1 receptors are abundantly expressed within the limbic system (McPartland *et al*, 2007), particularly in the basolateral complex of the amygdala (BLA), hippocampus and mPFC (Herkenham *et al*, 1991; Tsou *et al*, 1998), where, by regulating synaptic plasticity (Katona and Freund, 2012), play a crucial role in the modulation of memory for emotionally arousing experiences (Atsak *et al*, 2012b; Campolongo *et al*, 2013; Campolongo *et al*, 2009; Ganon-Elazar and Akirav, 2009; Marsicano and Lafenetre, 2009; Marsicano *et al*, 2002; Morena and Campolongo, 2013; Tan *et al*, 2011; Wotjak, 2005).

It is well known that stress hormones, released after an emotionally arousing event, interact with the endocannabinoid system. It has been shown that cannabinoids modulate noradrenergic activity (Muntoni *et al*, 2006; Oropeza *et al*, 2005), stimulate glucocorticoid secretion (Hill and McEwen, 2010b; Hill *et al*, 2010c; Steiner and Wotjak, 2008) and mediate some of the central effects induced by glucocorticoids (Atsak *et al*, 2012a; Atsak *et al*, 2012b; Barna *et al*, 2004;

Morena *et al*, 2013; Weidenfeld *et al*, 1994)}. On the other hand, both stress and glucocorticoids regulate limbic endocannabinoid response, thus demonstrating that cannabinoids and glucocorticoids are mutually regulated (Hill *et al*, 2010b). It is well established that stress hormones play an important role in the regulation of memory consolidation for emotionally arousing experiences (Roozendaal *et al*, 2009a), a cognitive process that require the selective activation of the BLA (Roozendaal and McGaugh, 1996; Roozendaal *et al*, 2006). We and others have previously showed that the amygdalar endocannabinoid signaling mediates emotional arousal influence on memory modulation (Campolongo *et al*, 2009; Marsicano *et al*, 2002; Morena *et al*, 2013). In particular, we previously reported that the cannabinoid agonist WIN55,212-2, bilaterally infused into the BLA immediately after inhibitory avoidance training, enhanced memory consolidation. Conversely, the CB1 receptor antagonist AM251 administered after training into the BLA dose-dependently impaired 48-h inhibitory avoidance retention (Campolongo *et al*, 2009). It is well established that the BLA plays an important role in integrating stress modulatory influences on memory consolidation of different kinds of information through its many efferent projections to other brain regions (McGaugh, 2000). The BLA, indeed, projects both directly and indirectly to the hippocampus (Petrovich *et al*, 2001; Pikkarainen *et al*, 1999), and there is considerable evidence that interactions between the BLA and the hippocampus regulate emotional-arousal effects on memory consolidation of spatial or contextual information (Huff *et al*, 2006; Roozendaal and McGaugh, 1997; Roozendaal *et al*, 1999). In addition to the hippocampus, the BLA has been extensively demonstrated to interact with the mPFC in modulating memory acquisition (Laviolette and Grace, 2006; Tan *et al*, 2011) and consolidation (Roozendaal *et al*, 2009b) for emotionally arousing trainings.

This evidence prompted us to hypothesize that, after an emotionally arousing experience, endocannabinoids might be released within the BLA and other limbic regions to modulate the storage of emotionally salient events. Therefore, in the present study we first investigated whether endocannabinoid contents in limbic areas is modified during early phases of memory consolidation for emotional events. To this aim parallel groups of rats were trained in an inhibitory avoidance task under three different experimental conditions (i, exposed to the context in the absence of any footshock; ii, trained with a 0.35 mA footshock; iii, trained with a 0.45 mA footshock). We then evaluated AEA and 2-AG content in the amygdala, hippocampus and mPFC 10, 30 or 60 minutes after exposure to the context or

training. In a second experiment we used a pharmacological approach to investigate whether endogenous released cannabinoids play any role in the consolidation of memory for emotionally arousing inhibitory avoidance training. Different doses of the FAAH enzyme inhibitor URB597, which increases AEA levels, were administered into the BLA, hippocampus or mPFC immediately after an aversively motivated inhibitory avoidance training. To investigate whether the memory effect of URB597 could be mediated by activation of CB1 receptors, in a separate experiment, animals were concurrently infused with URB597 and a non-impairing dose of the CB1 receptor antagonist AM251 immediately after the training trial. Memory retention was tested 48-h after the training trial.

To explore the hypothesis that the BLA may modulate endocannabinoid response to aversive training experiences in the hippocampus and mPFC, in another experiment we selectively induced bilateral permanent lesions of the BLA and measured endocannabinoid levels in the hippocampus and mPFC in rats subjected to an inhibitory avoidance training. Finally, in the last experiment, we infused URB597 into the hippocampus or the mPFC immediately after the training trial in rats with bilateral permanent lesions of the BLA and tested memory retentions 48-h after training and drug administration.

Thus, the present study has a two-fold interest: (1) to investigate the role of the endocannabinoid system within the BLA, hippocampus and mPFC in the modulation of memory consolidation for emotionally arousing experiences; (2) to examine the role of the BLA in coordinating endocannabinoid response to stressful events in the hippocampus and mPFC.

Materials and Methods

Animals. Male adult Sprague-Dawley rats (320-370 g at the time of behavioral experiments; Charles River Laboratories, Calco, Italy) were individually housed in a temperature-controlled (20±1°C) vivarium and maintained under a 12 h light/dark cycle (07:00 AM-07:00 PM h lights on). Food and water were available *ad libitum*. Training and testing were performed during the light phase of the cycle between 10:00 AM to 04:00 PM. All experimental procedures were in compliance with the guidelines of the U.S. National Institutes of Health and the Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004).

Surgery. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and were subsequently injected with 3 ml of saline (s.c.) to facilitate clearance of these drugs and prevent dehydration. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), and 2 stainless-steel guide cannulae (23 gauge) were implanted bilaterally, with the cannula tips 2 mm above the BLA [15 mm; coordinates: anteroposterior (AP), -2.8 mm from bregma; mediolateral (ML), ±5.0 mm from the midline; dorsoventral (DV), -6.5 mm from skull surface] or 1.5 mm above the CA1 region of the dorsal hippocampus [11 mm; coordinates: AP, -3.4 mm; ML, ±1.8 mm; DV, -2.7 mm] or 1.5 mm above the prelimbic region of the mPFC [11 mm; coordinates: AP, +3.7 mm; ML, ±0.7 mm; DV, -2.4 mm] (Atsak *et al*, 2012a; Roozendaal *et al*, 2009b)according to the atlas of Paxinos and Watson (Paxinos and Watson, 2005). The cannulae were affixed to the skull with 2 anchoring screws and dental cement. Stylets (15- or 11-mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency. Other groups of rats received bilateral NMDA-induced neurotoxic lesions of the BLA (Sigma-Aldrich, Milan, Italy; 1.25 mg per 100 µl of phosphate buffer, pH 7.4) alone or in addition to bilateral cannulae in the hippocampus or in the mPFC (Roozendaal *et al*, 2003). The NMDA solution was backfilled into a 10µl Hamilton microsyringe (30 gauge needle), driven by an intergrated stereotaxic minipump (Stoelting Co., Varese, Italy). The microsyringe needle was inserted into the BLA (coordinates: AP, -2.8 mm; ML, ±5.0 mm; DV, -8.5 mm), and NMDA (2.5 µg per 0.2 µl of phosphate buffer) was infused over a 30 s period. The injection needle was retained in place for an additional 3 min to optimize diffusion. Sham operations used the same procedure except that the needle was lowered only to the level of the caudate/putamen (coordinates: AP, -2.8 mm; ML, ±5.0 mm; DV, -6.5 mm) and removed after 3 min without infusion. After surgery, the rats were retained in an incubator until recovered from anesthesia and were then returned to their home cages. Rats were allowed to recover from surgery for 10 days before training. The rats were handled 1 min per day for 3 days before training.

Inhibitory avoidance apparatus and experimental procedures. For all experiments, rats were trained in an inhibitory avoidance apparatus consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh *et al*, 1988). The starting

compartment (31 cm) was made of opaque white plastic and illuminated by a lamp; the shock compartment (60 cm) was made of dark, electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound- and light-attenuated room. For training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to freely explore the apparatus. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.35-0.6 mA) was delivered. Rats were removed from the shock compartment 15 s later and returned to their home cages. Retention was tested 48-h after training by placing the rat into the starting compartment of the inhibitory avoidance apparatus and by measuring the latency to enter the shock compartment with all four paws (maximum latency of 600 s). Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial. Immediately after the training and testing of each animal, the apparatus was cleaned with a 70% ethanol solution. In the first experiment non-cannulated rats were trained without the exposure to any footshok (No FS), to 0.35 mA (Low FS) or to 0.45 mA (High FS) footshocks. Parallel groups of rats were then either tested for 48-h retention latencies or sacrified at different time points after the training trial (10, 30 or 60 min) for brain tissue dissection. In the other experiments cannulated- and BLA-lesioned animals were subjected to the behavioral training described above (FS 0.6-0.8 mA).

Drug treatment. The FAAH inhibitor URB597 was administered into either the BLA (3, 10 or 30 ng in 0.2 µl), hippocampus or mPFC (3, 10 or 30 ng in 0.5 µl). Each dose was chosen on the basis previous pilot experiments conducted in our laboratory. To examine whether the effects of URB597 are mediated *via* indirect anandamide activation of CB1 receptors, in other groups of rats the effective dose of URB597 (10 ng in 0.2 µl for BLA, 10 ng in 0.5 µl for Hipp or 30 ng in 0.5 µl for mPFC) was infused either alone or concurrently with a non-impairing dose of the CB1 receptor antagonist AM251 (0.14 ng in 0.2 µl for BLA, 0.28 ng in 0.5 µl for Hipp and mPFC). In a third experiment, bilateral BLA sham or lesioned rats were infused with the effective dose of URB597 either into the Hipp (10 ng in 0.5 µl) or into the mPFC (30 ng in 0.5 µl). All drugs were dissolved in a vehicle containing 5% Polyethylene glycol, 5% Tween80 and 90% saline and administered into the BLA or hippocampus or mPFC immediately after inhibitory avoidance training. Bilateral infusions of drugs or an equivalent volume of vehicle into these brain regions

were given immediately after inhibitory avoidance training by using a 30 gauge injection needle connected by polyethylene tubing (PE-20) to a 10 µl Hamilton microsyringe driven by a minipump (KD Instruments, Varese, Italy). For BLA infusions, the injection needle protruded 2.0 mm beyond the tip of the cannula and a 0.2 µl injection volume/hemisphere was infused over a period of 25 s. For hippocampus and mPFC infusions, the injection needles protruded 1.5 mm beyond the cannula tip and a 0.5 µl injection volume/hemisphere was infused over a period of 50 s. The injection needles were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volumes were chosen on the basis of our previous experiments (Malin and McGaugh, 2006; Roozendaal *et al*, 1996, 1997). All drugs were purchased from Tocris Bioscience (Milan, Italy) and freshly prepared before each experiment.

Histology. Rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with a 0.9% saline solution. The brains were then removed and immersed in a 4% formaldehyde solution. At least 48-h before sectioning, the brains were transferred to a 20% sucrose solution in saline for cryoprotection. Coronal sections of 35 µm were cut on a cryostat, mounted on gelatin coated slides, and stained with cresyl violet. The sections were examined under a light microscope (Microscope Nikon 801, Florence, Italy) and the location of infusion needle tips in the BLA, hippocampus or mPFC as well as the determination of the size and location of the lesions of the BLA was made according to the standardized atlas plates of Paxinos and Watson (Paxinos *et al*, 2005) by an observer blind to drug treatment condition. For all experiments, only rats with needle tips within the boundaries of the BLA or hippocampus or mPFC were included in the data analysis. Approximately 15% of the animals were excluded from analysis because of either cannula misplacement or damage to the targeted tissue. For the excitotoxic lesion experiments, approximately 17% of the animals with misplaced or absent lesions were excluded from further analysis.

Endocannabinoid extraction and analysis. AEA and 2-AG endocannabinoid levels in the amygdala, hippocampus and mPFC were measured 10, 30 or 60 min after training on non-cannulated rats trained under different inhibitory avoidance experimental conditions (0.00 mA, 0.35 mA, 0.45 mA). After rapid decapitation, the amygdala, hippocampus and mPFC were dissected within 3 min. The brain tissue was collected and stored at -80°C. Before the extraction process, tissues

were weighted and homogenized in polypropylene tubes (Sarstedt, Numbrecht, Germany) and kept in ice water. Five hundred µl of the described homogenized tissue solution was transferred to a 2-ml Eppendorf tube, and 20 µl of internal standard and 1 ml methyl tertiary butyl ether (Sigma-Aldrich, Milan, Italy) were added to extract the endocannabinoids. The mixture was vortexed for 1 min and centrifuged at 12,000g for 6 min at 4 °C. The clear supernatant was transferred into a clean 5-ml polypropylene tube (Sarstedt, Numbrecht, Germany) and evaporated under vacuum at 37°C. The residue of all evaporated samples was reconstituted in 100 µl acetonitrile, vortexed for 30 s, and sonicated in 4°C water for 15 min. A 20-µl aliquot of the clear solution was used for isotope-dilution liquid chromatography-tandem mass spectrometry analysis as described previously (Hauer *et al*, 2010; Schelling *et al*, 2006). All samples were injected in duplicates.

Statistics. Data were analyzed with one- or two-way ANOVAs. The source of the detected significances was determined by Tukey–Kramer *post hoc* tests. To determine whether learning had occurred, paired t tests were used to compare the training and retention latencies of the vehicle groups. Unpaired t test was used to compare training latencies of sham lesioned and BLA lesioned groups. Data are expressed as mean ± SEM. *P* values of less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

Results

Emotionally arousing inhibitory avoidance training increases AEA levels within the amygdala, hippocampus and mPFC only under high aversive conditions, without altering 2-AG levels

To investigate whether the level of emotional arousal influenced long-term memory retention, parallel groups of rats were trained in an inhibitory avoidance task under three different conditions (No FS; Low FS or High FS). Average step-through latencies for all groups during training, before footshock, were 14.2 ± 1.9 s. One-way ANOVA for training latencies revealed no significant differences between groups ($F_{2,17}$ = 0.08; *P* = 0.92; Fig. 1A). As shown in Fig. 1B, different levels of footshock intensity progressively increased the memory retention. A one-way ANOVA for retention latencies revealed a significant footshock condition

effect ($F_{2,17}$ = 20.41; P < 0.0001). *Post hoc* comparisons indicated that retention latencies of rats given the high footshock intensity (0.45 mA) were significantly longer than those of both rats only exposed to the context without any footshock (P < 0.01) and rats given the low footshock intensity (0.35 mA; P < 0.05). Rats given the low footshock intensity had higher retention latencies than rats only exposed to the experimental apparatus (P < 0.05).

Next, we examined whether an emotionally arousing inhibitory avoidance training trial affected endocannabinoid AEA and 2-AG tissue levels within the amygdala, hippocampus and mPFC. Rats were trained in an inhibitory avoidance task under three different conditions (No FS; Low FS or High FS) and sacrified 10, 30 or 60 min after the training trial for brain dissection and subsequent endocannabinoid measurements. As is shown in Fig 2A a two-way ANOVA for AEA levels revealed a significant footshock condition effect ($F_{2,50}$ = 16.92; P < 0.0001), but no time point effect ($F_{2,50}$ = 0.003; P = 0.99) or interaction between both factors ($F_{4,50}$ = 0.40; P = 0.81). *Post hoc* comparisons indicated that the highest footshock intensity significantly increased amygdalar AEA levels 10, 30 and 60 min after the training trial as compared to rats only exposed to the experimental

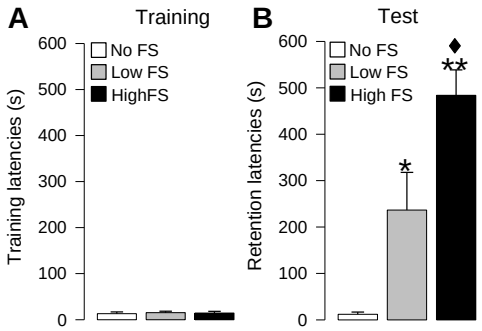

Figure 1. Effect of emotional arousal on inhibitory avoidance memory retention. (A) Step-through latencies on inhibitory avoidance training trial of rats trained under three different conditions (only exposed to the context without receiving any footshock, No FS; with a low footshock intensity, Low FS; with a high footshock intensity, High FS). (B) Step-through latencies (mean and SEM) on a 48-h retention test. The increase of footshock intensity during the training trial enhanced memory consolidation of the inhibitory avoidance task. *, P < 0.05, ** P < 0.01 vs No FS group; ◆, P < 0.05 vs Low FS group. Results represent mean ± SEM (n = 6-7 per group).

context (P < 0.05, for 10- and 30-min time points; P < 0.01, for 60-min time point) and to rats given the lower footshock intensity (P < 0.05 for 10- and 60-min time points; P < 0.01 for 30-min time point). As is shown in Fig 2B a two-way ANOVA for AEA levels into the hippocampus revealed a significant footshock condition

effect ($F_{2,44}$ = 14.25; P < 0.0001), no time point effect ($F_{2,44}$ = 1.27; P = 0.29) and a significant interaction between both factors ($F_{4,44}$ = 2.73; P = 0.041). *Post hoc* comparisons indicated that the highest footshock intensity significantly increased hippocampal AEA levels 10 min after the training trial as compared to both rats only exposed to the experimental context and rats given the low footshock intensity (P < 0.01 for both comparisons). A two-way ANOVA for AEA levels into the mPCF revealed a significant footshock condition effect ($F_{2,49}$ = 5.47; P < 0.0072), but no time point effect ($F_{2,49}$ = 2.94; P = 0.062) or an interaction between both factors ($F_{4,49}$ = 1.54; P = 0.20; Fig. 2C). *Post hoc* comparisons indicated that the highest footshock intensity significantly increased AEA levels into the mPFC 60 min after the training trial as compared to rats only exposed to the experimental

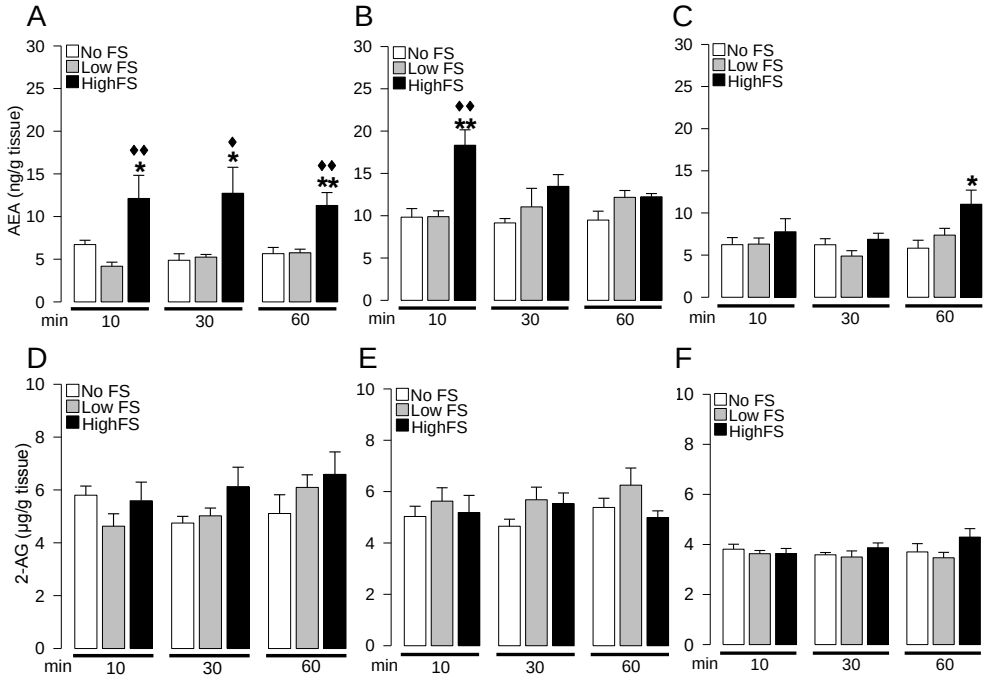

Figure 2. Effect of the level of emotional arousal of an inhibitory avoidance training trial on endocannabinoid AEA and 2-AG levels into the amygdala, hippocampus and mPFC, measured at different time points during memory consolidation. The high footshock (High FS) condition induced an increase in AEA levels into the amygdala (A) 10, 30 and 60 min after the training, into the hippocampus (B) 10 min after the training and into the mPFC (C) 60 min after the training, without altering 2-AG content neither into the amygdala (D), nor into the hippocampus (E), nor into the mPFC (F). *, P < 0.05, **, P < 0.01 vs the correspondent no footshock (No FS) group; ◆, P < 0.05, ◆◆, P < 0.01 vs the correspondent low footshock (Low FS) group. All results represent mean ± SEM (n = 3 - 8 per group).

context ($P < 0.05$; Fig. 3B). Inhibitory avoidance training experience did not affect 2-AG levels neither into the amygdala (Fig. 2D) nor into the hippocampus (Fig. 2E) or into the mPFC (Fig. 2F). A two-way ANOVA for 2-AG levels revealed that there was neither a footshock condition effect ($F_{2,50} = 2.10$; $P = 0.13$; $F_{2,43} = 2.78$; $P = 0.073$; $F_{2,46} = 1.69$; $P = 0.20$; for amygdala, hippocampus and mPFC, respectively) nor a time point effect ($F_{2,50} = 1.03$; $P = 0.36$; $F_{2,43} = 0.34$; $P = 0.71$; $F_{2,46} = 0.39$; $P = 0.68$; for amygdala, hippocampus and mPFC, respectively), nor an interaction between both factors ($F_{4,50} = 1.10$; $P = 0.37$; $F_{4,43} = 0.77$; $P = 0.55$; $F_{4,46} = 0.69$; $P = 0.60$; for amygdala, hippocampus and mPFC, respectively). Thus, these findings indicate that the level of emotional arousal at encoding dose-dependently enhanced memory retention and that high arousal training conditions selectively increased AEA levels at different time points after the aversive experience within the amygdala, hippocampus and the mPFC, without affecting 2-AG levels.

Posttraining infusions of the FAAH inhibitor URB597 into the BLA, hippocampus and mPFC induce enhancement of inhibitory avoidance retention *via* indirect activation of CB1 receptors

This experiment examined whether posttraining bilateral intra-BLA, intra-hippocampal or intra-mPFC infusions of the FAAH inhibitor URB597 immediately after inhibitory avoidance training modulated memory retention of this trial. Average step-through latencies for all groups during trainings, before footshock and drug treatment, were 13.8 ± 0.9 s, 14.4 ± 1.2 s and 16.4 ± 1.3 s for BLA, hippocampus and mPFC, respectively. One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,46} = 1.31$; $P = 0.28$; $F_{3,32} = 1.05$; $P = 0.38$; $F_{3,49} = 0.47$; $P = 0.71$; for BLA, hippocampus and mPFC, respectively). Forty-eight-hour retention latencies of rats infused with vehicle into the BLA, hippocampus or into the mPFC immediately after training were significantly longer than their entrance latencies during the training trial ($t_{11} = -3.63$; $P = 0.0039$; $t_{10} = -2.34$; $P = 0.042$ and $t_{12} = -2.51$; P = 0.028, respectively), indicating that the rats retaine memory of the shock experience. As shown in Fig. 3A-B, URB597 infused into the BLA or hippocampus improved 48-h memory retention in an inverted U-shape relationship. One-way ANOVAs for retention latencies revealed a significant treatment effect ($F_{3,46} = 3.63$; $P = 0.020$; $F_{3,32} = 3.30$; $P = 0.033$, for BLA and hippocampus, respectively). *Post hoc* comparisons indicated that retention latencies of rats given posttraining infusions of URB597 (10 ng) were significantly longer than those of rats given vehicle ($P < 0.05$, for both

comparisons; Fig. 3A-B). Lower or higher doses (3 ng and 30 ng) did not significantly affect retention performance. Figure 3C shows that URB597 enhanced 48-h memory retention when infused into the mPFC. A one-way ANOVA for retention latencies revealed a significant treatment effect ($F_{3,49}$ = 3.67; P = 0.018). *Post hoc* comparisons indicated that retention latencies of rats given posttraining infusions of URB597 (30 ng) were significantly longer than those of rats given vehicle ($P < 0.05$). Lower doses (3 ng and 10 ng) did not significantly affect retention performance.

Next, we examined whether the memory enhancing effect induced by URB597 is mediated by the indirect activation of CB1 receptors. To address this question rats were concurrently infused into the BLA, hippocampus or mPFC with the effective dose of URB597 together with a non-impairing dose of the CB1 antagonist AM251 immediately after the training trial. Average step-through latencies for all groups during trainings, before footshock and drug treatment, were 10.0 ± 0.9 s, 12.2 ± 0.8 s and 15.6 ± 1.3 s for BLA, hippocampus and mPFC, respectively. Two-way ANOVAs for training latencies revealed no significant differences between posttraining drug groups (for all comparisons $P > 0.11$). Forty-eight-hour retention latencies of rats infused with vehicle into the BLA, hippocampus or mPFC immediately after training were significantly longer than their entrance latencies during the training trial (t_9 = -2.89; P = 0.018; t_{12} = -2.23; P = 0.046 and t_9 = -2.34; P = 0.044, respectively), indicating that the rats retained memory of the shock experience. Fig. 3D shows retention latencies of rats infused concurrently with URB597 and AM251 into the BLA immediately after training. A two-way ANOVA for memory retention revealed a significant URB597 treatment effect ($F_{1,34}$ = 5.47; P = 0.025), a significant AM251 treatment effect ($F_{1,34}$ = 4.27; P = 0.046) and a significant interaction between both factors ($F_{1,34}$ = 5.45; P = 0.026). *Post hoc* comparisons indicated that retention latencies of rats given posttraining infusions of URB597 (10 ng) were significantly longer than those of rats given vehicle ($P < 0.05$). Retention latencies of rats given a non-impairing dose of AM251 (0.14 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone ($P < 0.05$). Fig. 3E shows retention latencies of rats infused concurrently with URB597 and AM251 into the hippocampus immediately after training. A two-way ANOVA for memory retention revealed a significant URB597 treatment effect ($F_{1,47}$ = 8.42; P = 0.0056), a significant AM251 treatment effect ($F_{1,47}$ = 11.13; P = 0.0017) and a significant interaction between both factors

($F_{1,47}$ = 10.74; *P* = 0.0020). *Post hoc* comparisons indicated that retention latencies of rats given posttraining infusions of URB597 (10 ng) were significantly longer

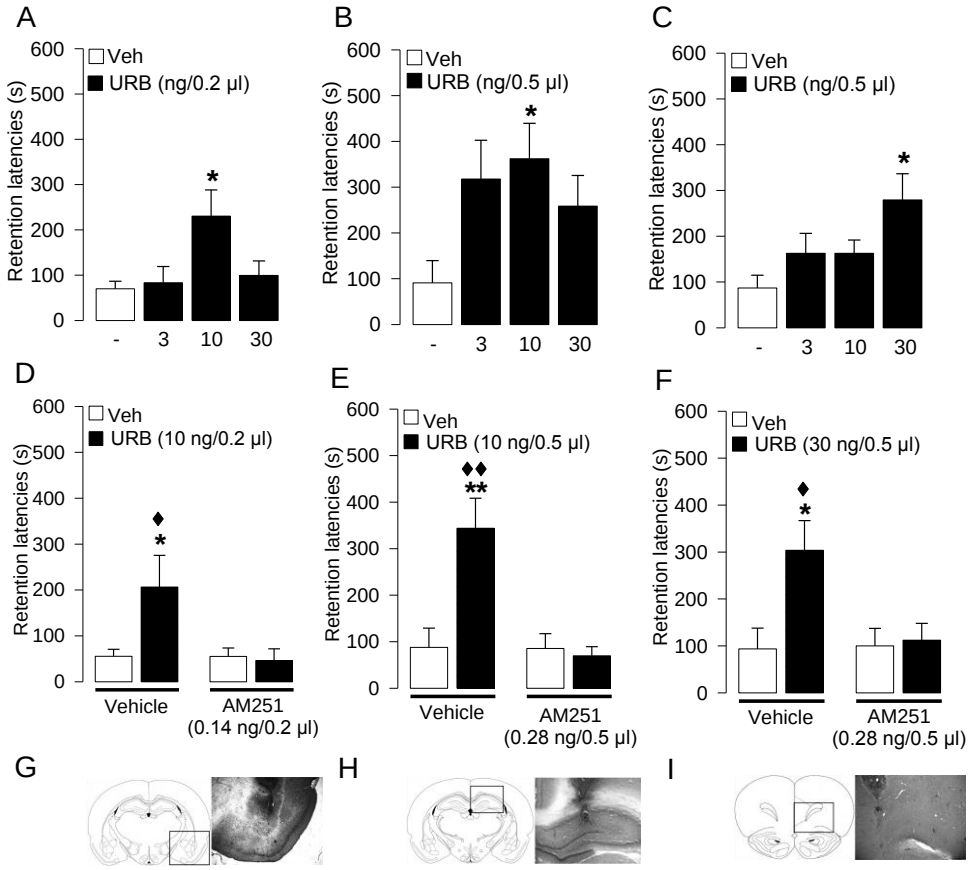

Figure 3. Effects of intra-BLA, intra-hippocampus or intra-mPFC infusions of the FAAH inhibitor URB597, either alone or together with the CB1 antagonist AM251, on 48-h memory retention. Immediate posttraining bilateral infusions of URB597 (URB; 3, 10 or 30 ng/0.5 µl) into the BLA (A), hippocampus (B) or mPFC (C) enhanced memory consolidation. Immediate posttraining bilateral infusions of a non-impairing dose of AM251 (0.14-0.28 ng/0.5 µl) blocked the memory-enhancing effects of concurrently administered URB597 (URB; 10 or 30 ng/0.5 µl) into the BLA (D), hippocampus (E) or into the mPCF (F). Representative photomicrograph (Microscope Nikon 801, original magnification 2X) illustrating placement of cannula and needle tip in the BLA (G), dorsal hippocampus (H) and in the mPFC (I). *, *P* < 0.05, **, *P* < 0.01 vs the corresponding vehicle (Veh) group; ♦, *P* < 0.05, ♦♦, *P* < 0.01 vs the corresponding AM251 group. All results represent mean ± SEM (n = 8 - 14 per group).

than those of rats given vehicle (*P* < 0.01). Retention latencies of rats given a non-impairing dose of AM251 (0.28 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone (*P* < 0.01). Fig. 3F shows retention latencies of rats infused concurrently with URB597 and AM251 into the

mPFC immediately after training. A two-way ANOVA for memory retention revealed a significant URB597 treatment effect ($F_{1,38}$ = 5.58; *P* = 0.023), no significant AM251 treatment effect ($F_{1,38}$ = 3.87; *P* = 0.056) and a significant interaction between both factors ($F_{1,38}$ = 4.41; *P* = 0.042). *Post hoc* comparisons indicated that retention latencies of rats given posttraining infusions of URB597 (30 ng) were significantly longer than those of rats given vehicle (*P* < 0.05). Retention latencies of rats given a non-impairing dose of AM251 (0.28 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone (*P* < 0.05). These results indicated that the memory enhancement induced by URB597 is mediated by an indirect activation of CB1 receptors into the BLA, hippocampus and mPFC.

Bilateral permanent lesions of the BLA block the training-induced increase of AEA levels within the hippocampus and the mPFC

The present study evaluated whether the BLA modulates endocannabinoid response to aversive training experiences in the hippocampus and mPFC. To explore this possibility, we selectively induced bilateral permanent lesions of the BLA and measured AEA and 2-AG endocannabinoid levels in the hippocampus and mPFC in rats subjected to an inhibitory avoidance training. Rats were trained under the 0.45 mA FS condition and sacrified 10 or 60 min later for hippocampus and mPCF dissection, respectively (in the previous experiments we found that AEA levels increased 10 and 60 min after training in the hippocampus and mPFC, respectively; see Fig. 2B-C). Fig 4A-B show the effects induced by BLA lesions on hippocampal and mPFC AEA levels. One-way ANOVAs for AEA levels into the hippocampus or into the mPFC revealed a significant experimental condition effect ($F_{2,14}$ = 25,04; *P* < 0.0001; $F_{2,16}$ = 5,58; *P* < 0.015 for hippocampus and mPFC, respectively). *Post hoc* comparisons indicated that bilateral BLA permanent lesions significantly abolished the increase in AEA levels seen in sham lesioned controls (*P* < 0.01; *P* < 0.05 for hippocampus and mPFC, respectively). Bilateral BLA lesions did not affect 2-AG levels neither into the hippocampus nor into the mPFC of rats subjected to the inhibitory avoidance training ($F_{2,14}$ = 0.16; *P* < 0.85; $F_{2,16}$ = 0.14; *P* < 0.87, respectively; data not shown). Thus, these findings indicate that bilateral lesions of BLA blocked the training-induced increase of AEA levels without affecting 2-AG levels.

Bilateral permanent lesions of the BLA block the memory enhancing effects induced by posttraining URB597 infusions in the hippocampus or in the mPFC

The present experiments evaluated the BLA modulates the memory effect induced by intra-hippocampus or intra-mPFC infusions of URB597. The first experiment examined the effects of URB597 infusions into the hippocampus of rats with bilateral BLA lesions. Average step-through latencies of rats with sham or BLA lesions to enter the shock compartment on the training trial, before footshock exposure, were not statistically different (12.1 ± 1.3 s for sham-lesioned rats, 10.2 ± 1.0 s for BLA lesioned rats; t_{48} = 1.17; P = 0.25). Two-way ANOVA for training latencies revealed no significant BLA lesion effect ($F_{1,46}$ = 1.46; P = 0.23), no differences between posttraining intra-hippocampal drug infusions ($F_{1,46}$ = 0.28; P = 0.60) or an interaction between both factors ($F_{1,46}$ = 0.68; P = 0.41). Forty-eight-hour retention latencies of sham lesioned rats infused with vehicle into the hippocampus were significantly longer than their entrance latencies during the training trial (t_{13} = -2.26; P = 0.041), indicating that rats retained memory for the task. As shown in Fig. 4C bilateral BLA lesions blocked the memory enhancing effect induced by URB597 infused into the hippocampus immediately after the training trial. Two-way ANOVA for retention latencies revealed a significant BLA lesion effect ($F_{1,46}$ = 12.10; P = 0.0011), a significant URB597 treatment effect ($F_{1,46}$ = 4.12; P = 0.048) and a significant interaction between these two factors ($F_{1,46}$ = 4.84; P = 0.033). Post-training infusions of URB597 (10 ng) into the hippocampus of sham lesioned rats enhanced 48-h retention of rats relative to their corresponding group (P < 0.05). BLA lesions alone, while not impairing retention memory, blocked the memory enhancing effect of URB597 infused into the hippocampus (P < 0.01). The second experiment examined the effects of URB597 infusions into the mPFC of rats with bilateral BLA lesions. Latencies of rats with sham lesions or BLA lesions to enter the shock compartment on the training trial, before footshock exposure, did not differ (15.4 ± 2.0 s for sham lesioned rats, 12.1 ± 1.5 s for BLA lesioned rats; t_{43} = 1.35; P = 0.18). Two-way ANOVA for training latencies revealed no significant BLA lesion effect ($F_{1,41}$ = 1.99; P = 0.17), no differences between posttraining intra-mPFC drug infusions ($F_{1,41}$ = 1.04; P = 0.31) or an interaction between both factors ($F_{1,41}$ = 0.46; P = 0.50). Forty-eight-hour retention latencies of sham lesioned rats infused with vehicle into the mPFC were significantly longer than their entrance latencies during the training trial (t_{10} = -2.68; P = 0.023), indicating that rats retained memory

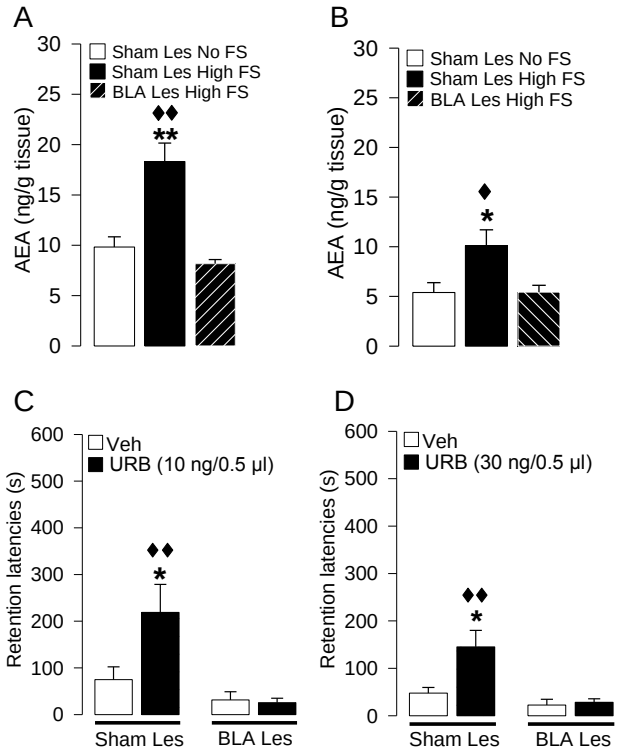

Figure 4. Role of the BLA in modulating endocannabinoid response to an emotionally arousing inhibitory avoidance training in the hippocampus and the mPFC. Bilateral permanent lesions of the BLA blocked the training-induced increase of AEA levels into the hippocampus (A) and mPFC (B), 10 and 60 min after the training trial, respectively. *, P < 0.05, **, P < 0.01 vs the corresponding sham lesioned group only exposed to the experimental context (Sham les No FS); ♦, P < 0.05, ♦♦, P < 0.01 vs the corresponding BLA lesioned group trained under the high footshock condition (Sham les High FS). Results represent mean ± SEM (n = 4 - 7 per group). Bilateral lesions of the BLA blocked the memory enhancing effects induced by URB597 infused into the hippocampus (10 ng/0.5 µl; C) or into the mPFC (30 ng/0.5 µl; D). *, *P* < 0.05 vs the corresponding vehicle group (Veh); ♦♦, *P* < 0.01 vs the corresponding BLA lesioned group (BLA les). Results represent mean ± SEM (n = 10 - 14 per group).

for the task. Fig. 4D shows that bilateral BLA lesions blocked the memory enhancing effect induced by URB597 infusions into the mPFC. Two-way ANOVA for retention latencies revealed a significant BLA lesion effect ($F_{1,41}$ = 11.80; *P* = 0.0014), a significant URB597 treatment effect ($F_{1,41}$ = 6.26; *P* = 0.016) and a significant interaction between these two factors ($F_{1,41}$ = 4.91; *P* = 0.032). *Post hoc* comparisons indicated that posttraining infusions of URB597 (30 ng) into the mPFC of sham lesioned rats enhanced 48-h retention of rats as compared to their corresponding vehicle control group (*P* < 0.05). Importantly, BLA lesions alone did

not impair retention latencies but blocked the memory enhancing effect of URB597 infused into the mPFC ($P < 0.01$). Thus, our findings indicated that the BLA is critically involved in mediating endocannabinoid enhancing effects in the hippocampus and mPFC on memory consolidation for the inhibitory avoidance training.

Discussion

The present findings demonstrate that the endogenous cannabinoid AEA crucially modulates the consolidation of memory for aversive experiences within the BLA, hippocampus and mPFC, three brain regions importantly involved in emotional memory processes (Price *et al*, 2010). Most importantly, our findings indicate that the BLA plays a crucial role in the coordination of the hippocampal and prefrontal cortex endocannabinoid response after a stressful event, shedding light on the dynamic interplay occurring among these three brain regions.

Here we show that rats trained under higher arousal conditions retain better memories of the event than rats trained to mild footshock intensities. Interestingly, this effect is paralleled by an increase of AEA levels in the BLA, hippocampus and mPFC, while 2-AG levels are unaffected. The increase in AEA content shows a temporal-dependent response during the early phases of memory consolidation, with permanent increase of AEA in the BLA (from 10 to 60 min after training) and an activation of the endocannabinoid tone only 10 min after training in the hippocampus and 60 min after training in the mPFC. Interestingly, the exogenous activation of the endocannabinoid tone induced by bilateral posttraining infusions of the FAAH inhibitor URB597 into all the three considered brain regions induced an enhancement of 48-h inhibitory avoidance retention performance. This memory enhancing effect is mediated by indirect activation of CB1 receptors since the concurrent administration of a nonimpairing dose of the CB1 receptor antagonist AM251 is able to block such effect.

It is well known that CB1 receptors, highly expressed throughout the limbic system (Herkenham *et al*, 1991; McPartland *et al*, 2007; Tsou *et al*, 1998), modulate neuronal signaling and synaptic plasticity (Chevaleyre *et al*, 2006; Katona *et al*, 2012; Marsicano and Lutz, 2006), thus, regulating emotional behavior and memory processes related to emotionally arousing events (Atsak *et al*, 2012b; Campolongo *et al*, 2009; Ganon-Elazar *et al*, 2009; Marsicano *et al*, 2009; Marsicano *et al*, 2002; Tan *et al*, 2011; Wotjak, 2005). Unfortunately, with

regard to cannabinoid effects on memory consolidation a large number of studies report contradictive findings. Some authors showed that posttraining intra-hippocampal administration of cannabinoid agonists impair memory consolidation in several behavioral tasks (e.g. step-down inhibitory avoidance, step-through inhibitory avoidance, Morris water maze) (Jamali-Raeufy *et al*, 2011; Yim *et al*, 2008; Zarrindast *et al*, 2011) while other studies reported enhancing effects when cannabinoid agonists were infused either into the hippocampus (De Oliveira Alvares *et al*, 2008) or into the BLA (Campolongo *et al*, 2009). In addition to differences in dose, route of administration and timing of exposure, cannabinoid effects on memory function are strikingly dependent on the level of emotional arousal induced by the environmental context and by the stressfulness of the experimental condition employed in the different studies (Campolongo *et al*, 2013; Campolongo *et al*, 2012; Morena *et al*, 2013)}. In support of this view in the present study we demonstrated that only under high (and not low) aversive conditions (i.e. augmented footshock intensity) AEA is released within the three key brain regions involved in memory processes for emotional events. Our findings indicate that the pavlovian association between a physical stress (i.e. 0.45 mA footshock intensity) and the dark compartment of the inhibitory avoidance apparatus induces a selective release of the endocannabinoid AEA in the BLA, hippocampus and mPFC, brain areas crucially involved in the memory modulation of the aversive experience. This increase in the endocannabinoid response was not detectable when animals were subjected to milder foot-shock intensities, still able to induce a memory of the event but less stronger in term of emotional arousal. The present finding is in line with the result of de Oliveira Alvares and co-workers (2010) reporting that immediate posttraining infusion of the cannabinoid antagonist AM251 into the dorsal hippocampus impaired 24-h memory retention of a contextual fear conditioning paradigm only when conducted under high arousal conditions (i.e. high footshock intensity) (de Oliveira Alvares *et al*, 2010). Similarly, Bucherelli and coworkers (2006) reported that the blockade of the endogenous cannabinoid neurotransmission in the amygdala obtained by local infusions of the CB1 receptor antagonist AM251 impaired memory consolidation of a contextual fear conditioning (Bucherelli *et al*, 2006).

We then pharmacologically tested whether an exogenous amplification of the endocannabinoid neurotransmission in the three brain areas objective of the study could modulate memory consolidation for inhibitory avoidance training. Our results showed that the FAAH inhibitor URB597, which increases endogenous

levels of AEA, enhanced 48-h memory retention when infused into the BLA, hippocampus or mPFC. Interestingly, the URB597 memory enhancing effect was blocked by a concurrent infusion of AM251, demonstrating a CB1-mediated effect in all the three brain areas. Our results are consistent with previous findings demonstrating that intra-hippocampal administration of AEA or the cannabinoid antagonist AM251 enhanced or impaired, respectively, memory consolidation of a step-down inhibitory avoidance task (de Oliveira Alvares *et al*, 2005; De Oliveira Alvares *et al*, 2008). To our knowledge only one very recent evidence has been recently reported in the literature concerning cannabinoid effects on memory consolidation when directly infused into the mPFC. Kuhnert et al. (2013) demonstrated that intra-mPFC infusions of AM251 impaired memory consolidation of an aversively motivated behavioral task (Kuhnert *et al*, 2013), again corroborating the accordance with the present findings.

As highlightened above, we have previously demonstrated that endocannabinoid effects on cognitive functions are strictly dependent on the aversiveness of the environmental condition and on the level of emotional arousal at the time of testing. This prompted us to hypothesize that the interaction with stress hormones is of crucial importance in determining the modulatory effects of cannabinoid compounds on memory processes. The amygdala represents a key region for the association between environmental information with emotional significance for the processing of both negative and positive emotions (Aggleton, 1993; Baxter and Murray, 2002; Davis *et al*, 1994; Pape and Pare, 2010). It is well established that emotionally arousing training experiences increase BLA neuronal activity (Pelletier *et al*, 2005) and the selective activation of the BLA is of crucial importance for enabling emotional arousal influence on memory modulation. Indeed, lesions of the BLA block the memory enhancing effects of an inhibitory avoidance training induced by a systemic administration of the synthetic glucocorticoid dexamethasone (Roozendaal *et al*, 1996). Corroborating and extending these findings, we demonstrated that bilateral lesions of the BLA block URB597 hippocampal and mPFC memory enhancing effects. In a previous paper we have shown that administration of the CB1 receptor antagonist AM251 in the BLA blocks the ability of systemically administered corticosterone to facilitate memory consolidation of inhibitory avoidance training (Campolongo *et al*, 2009). These findings indicated, for the first time *in vivo*, that glucocorticoids recruit endocannabinoid signaling in the BLA to modulate aversive memory consolidation (Hill *et al*, 2010a; Hill *et al*, 2010b). Within the BLA, CB1 receptors are abundantly

expressed in GABAergic interneurons (Katona *et al*, 2001) where they have consistently been shown to suppress the release of GABA (Katona *et al*, 2001; Katona *et al*, 1999; Ohno-Shosaku *et al*, 2001). It has been reported that inhibition of GABAergic activity within the BLA enhances memory consolidation by increasing the release of norepinephrine (Hatfield *et al*, 1999). In view of this evidence, we previously proposed a model: After experiencing an emotionally arousing event, corticosterone, released from the adrenal gland, binds to a membrane bound receptor in the BLA to stimulate the synthesis of endocannabinoids; once in the synaptic cleft, endocannabinoids may inhibit GABA release by presynaptic terminals, thus leading to the disinhibition of norepinephrine release and increased noradrenergic activation of postsynaptic β-adrenoceptors, increasing the consolidation of aversive memories (Atsak *et al*, 2012b; Campolongo *et al*, 2009; Hill and McEwen, 2009; Morena *et al*, 2013). Our present findings add to this model by demonstrating for the first time the increase of AEA during early phases of memory consolidation. Thus, after a stressful event there is an endogenous release of AEA mediated by a non-genomic effect of corticosterone and AEA mediates the enhancement of memory consolidation for aversive experiences *via* the activation of CB1 receptors. However, the BLA is thought not as a locus for memory storage; presumably, following its activation, this brain area modulates memory consolidation by coordinating the activation of other brain regions (Packard and Wingard, 2004). There are two different ways by which the BLA may influence the activation of other brain areas. First, the BLA may exert this modulatory influences indirectly by increasing autonomic and humoral stress responses (Roozendaal, 2002; Roozendaal *et al*, 1992). Accordingly with this assumption, de Oliveira Alvares and coworkers demonstrated that blockade of endocannabinoids action by intra-hippocampal infusion of the CB1 receptor antagonist AM251 impaired memory consolidation in rats that had received dexamethasone immediately before training, thus demonstrating that blockade of hippocampal endocannabinoid transmission abolishes glucocorticoid effects on memory consolidation (de Oliveira Alvares *et al*, 2010). We can speculate that, as a consequence, stress hormones may interact with the increased levels of AEA into the hippocampus or the mPFC, thus enhancing memory consolidation. The second hypothesis with regard to BLA modulation of other brain areas in the regulation of memory consolidation, is that the BLA might modulate cognitive functions through either direct or indirect neural connections to various limbic structures (Krettek and Price, 1978; Pape *et al*, 2010; Petrovich *et*

al, 2001; Pikkarainen *et al*, 1999). In the present study we show for the first time that the enhancement of memory consolidation requires a release of amygdalar endocannabinoids and, most importantly, that a functional BLA is required to enable hippocampal and mPFC endocannabinoid effects on memory consolidation. This demonstrates a critical role for BLA CB1 neurotransmission in modulating projection neurons to other brain regions during the processing of emotionally salient information., There is considerable evidence corroborating our findings which demonstrates that interactions between the BLA and the hippocampus regulate emotional-arousal effects on memory consolidation of spatial or contextual information (Huff *et al*, 2006; Roozendaal *et al*, 1997; Roozendaal *et al*, 1999). For instance, pharmacological inactivation of the BLA impaired memory consolidation and decreased ARC mRNA and protein levels in the dorsal hippocampus (McIntyre *et al*, 2005). Other studies, indicated that an intact and functional BLA is required to enable memory modulation that is initiated by a manipulation of hippocampal activity. In particular, posttraining hippocampal infusions of a glucocorticoid receptor agonist enhanced memory consolidation of inhibitory avoidance training but, most importantly, inactivation of the BLA blocked this effect (Roozendaal *et al*, 1997; Roozendaal *et al*, 1999). In addition to the hippocampus, it has been extensively demonstrated that the BLA interacts with the mPFC in modulating memory acquisition (Laviolette *et al*, 2006; Tan *et al*, 2011) and consolidation (Roozendaal *et al*, 2009b) for emotionally arousing training experiences. Tan and coworkers (2011) demonstrated that the modulation of cannabinoid transmission specifically within the BLA strongly influences: i) neuronal firing frequency and burst activity in mPFC neuronal subpopulations, ii) the encoding of emotionally salient associative memories via functional connectivity with the mPFC (Tan *et al*, 2011). It has been demonstrated that glucocorticoid receptor agonist infusion into the mPFC after inhibitory avoidance training enhanced memory consolidation and induced a rapid increase in BLA levels of phosphorylated extracellular signal-regulated kinase 1/2 (pErk1/2) and *vice versa*, suggesting a bidirectional interaction between the BLA and the mPFC in the regulation of memory consolidation (Roozendaal *et al*, 2009b).

In addition to the evidence already present in the literature, our results further indicate that depending on the brain region considered, AEA increases with distinct temporal windows during early phases of memory consolidation. It is likely that the activation of the BLA drives the endocannabinoid response into the other two brain regions considered with a time-dependent fashion. Such evidence

was confirmed by the finding that permanent lesions of the BLA blocked the training-induced increase of AEA levels both into the hippocampus and the mPFC. Moreover, bilateral posttraining infusions of URB597 into the hippocampus or into the mPFC did not enhance memory retention in rats with permanent lesions of the BLA, thus confirming that the BLA is crucially involved in coordinating endocannabinoid response to an aversive event in the hippocampus and mPFC. It has been proposed that the initiation of a strong emotional experience activates memory-related neuroplasticity in the hippocampus and amygdala and suppresses PFC functioning (Diamond *et al*, 2007). Such model suggests a rapid activation of the BLA (from seconds to hours) and hippocampus (from seconds to minutes) after a stressful experience, followed by an inhibitory phase. Conversely, stress induces an inhibition of the functioning of the mPFC and the recovery from its suppression of functioning depends on the nature and intensity of the stressor. According with this model we found a rapid increase of AEA levels within the BLA (from 10 to 60 minutes after the FS) and the hippocampus (10 minutes after the FS). Such rapid increase of amygdalar AEA levels, may represent one of the most rapid action of its activation. The subsequent stimulation of BLA CB1 receptors may decrease feedforward inhibition via inhibitory interneurons, thereby increasing the activity of BLA projection neurons (Pistis *et al*, 2004). The emotionally arousal-induced activation of the BLA may serve as a marker warning the hippocampus to process and stabilize important events to be remembered. On the other hand, basal mPFC activity is known to provide inhibitory influence on BLA activity (Likhtik *et al*, 2005; Quirk and Gehlert, 2003a; Quirk *et al*, 2003b), whereas stress and glucocorticoids suppress mPFC functioning. As a consequence the BLA greater activation during emotionally arousing situations (Amat *et al*, 2005; Davidson, 2002) may then enhance the consolidation of different kinds of information *via* its projections to other brain regions.

Taken together our findings suggest that as a response to stress endocannabinoids are released within three important limbic regions where they modulate the consolidation of memory for emotionally arousing events. Immediately after an aversive experience, it occurs a temporal interplay between the BLA, hippocampus and the mPFC modulated by the endocannabinoid system. In this scenario it is plausible to hypothesize that the endocannabinoid system, particularly AEA within the BLA, drives the disinhibition of neuronal projections to the hippocampus first and subsequently to the mPFC with the final result of a

coordination of memory processes underlying the consolidation of emotionally arousing experiences.

These finding have important clinical implications. Understanding the neural underpinnings of the temporal interactions between limbic regions after experiencing a stressful event will shed light on the neural mechanism involved in psychiatric disorders such as the post-traumatic stress disorder (PTSD), thus leading to new therapeutic opportunities.

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CHAPTER 2

PROPOFOL ENHANCES MEMORY FORMATION *VIA* AN INTERACTION WITH THE ENDOCANNABINOID SYSTEM

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Abstract

Background: Propofol is associated with postoperative mood alterations and induces a higher incidence of dreaming compared with other general anesthetics. These effects might be mediated by propofol's inhibitory action on fatty acid amide hydrolase, the enzyme that degrades the endocannabinoid anandamide. Because propofol is also associated with a higher incidence of traumatic memories from perioperative awareness and intensive care unit treatment and the endocannabinoid system is involved in regulating memory consolidation of emotional experiences, the authors investigated whether propofol, at anesthetic doses, modulates memory consolidation *via* an activation of the endocannabinoid system.

Methods: Male Sprague-Dawley rats were trained on an inhibitory avoidance task in which they received an inescapable foot shock upon entering the dark compartment of the apparatus. Drugs were administered intraperitoneally immediately or 30, 90, or 180 min after training. On the retention test 48 h later, the latency to reenter the dark compartment was recorded and taken as a measure of memory retention.

Results: The anesthetic doses of propofol administered after training significantly increased latencies of 48-h inhibitory avoidance performance (483.4 ± 181.3, 432.89 ± 214.06, 300 and 350 mg/kg, respectively; mean ± SD) compared with the corresponding vehicle group (325.33 ± 221.22, mean ± SD), which is indicative of stronger memory consolidation in propofol treated rats. Administration of a nonimpairing dose of the cannabinoid receptor antagonist rimonabant blocked the memory enhancement induced by propofol (123.39 ± 133.10, mean ± SD). Delayed administration of propofol 90 and 180 min after training or immediate posttraining administration of the benzodiazepine midazolam or the barbiturate pentobarbital did not significantly alter retention.

Conclusions: These findings indicate that propofol, in contrast to other commonly used sedatives, enhances emotional memory consolidation when administered immediately after a stressful event by enhancing endocannabinoid signaling.

Introduction

Propofol is a commonly used agent for general anesthesia and for sedation in patients undergoing intensive care treatment (ICU). It is known to reduce postoperative nausea and vomiting (Apfel *et al*, 2004) and is associated with postoperative mood alterations and a higher incidence of dreaming compared with other general anesthetics. However, the use of propofol for general anesthesia or for sedation of critically ill patients in the ICU is not universally successful with respect to preventing traumatic memories from perioperative awareness and ICU treatment (Jones *et al*, 2007). There is extensive evidence that the occurrence of traumatic experiences associated with perioperative awareness or ICU treatment could result in stress-related disorders such as posttraumatic stress disorder and impaired long-term health-related quality of life outcomes (Kapfhammer *et al*, 2004; Schelling *et al*, 2003). One clinical study, investigating propofol's effects on memory, reported that propofol inhibits conscious memory processing in human subjects soon after memory encoding and that it impairs the encoding of material into long-term memory (Veselis *et al*, 2009). In another study, propofol administration to rats induced amnesia of training on an inhibitory avoidance task (Alkire *et al*, 2001). However, in both studies propofol was administered before learning, thus revealing propofol's effect on the encoding of new information. No studies are available regarding propofol's effects on the consolidation of traumatic memories. However, because patients often have experienced stressful events, such as preoperative fear and anxiety, car accidents, myocardial infarctions, or acute respiratory distress shortly before induction of general anesthesia or sedation with propofol, it is crucial to investigate the effects of propofol administered shortly after the acquisition of new information, a time window when the memory trace is consolidated into stable long-term memory.

Propofol inhibits the enzyme fatty acid amide hydrolase, which is known to degrade endocannabinoids, especially anandamide (Patel *et al*, 2003). Like propofol, the endocannabinoid system recently has been shown to be crucially involved in mood control in animals (Bortolato *et al*, 2006; Gobbi *et al*, 2005) and the regulation of nausea and vomiting in humans during stress (Chouker *et al*, 2010). Thus, some of the mentioned propofol effects could be attributable to an activation of the endocannabinoid system (Di Marzo, 2003). Propofol administration to mice has been shown to increase endocannabinoid content within the brain, an effect that could not be detected with other sedative agents, such as midazolam or thiopental (Patel *et al*, 2003). In addition, endocannabinoid

plasma concentrations increased moderately in patients undergoing propofol anesthesia but decreased in patients undergoing general anesthesia with a volatile agent such as sevoflurane (Schelling *et al*, 2006) or isoflurane (Weis *et al*, 2010). The endocannabinoid system consists of endocannabinoid ligands, the endogenous cannabinoid receptors 1 and 2 (CB1 and CB2), and enzymes involved in the synthesis and metabolism of endocannabinoids (De Petrocellis and Di Marzo, 2009). Endocannabinoids (*i.e.*, anandamide and 2-arachidonoylglycerol) are synthesized on demand through cleavage of membrane precursors and serve as retrograde messengers at central synapses (Hashimotodani *et al*, 2007). They bind to CB1 receptors on axon terminals to regulate ion channel activity and neurotransmitter release (Piomelli, 2003) and are degraded intracellularly by specific enzymes: anandamide is mainly degraded by fatty acid amide hydrolase and 2-arachidonoylglycerol by monoacylglycerol lipase (Petrosino and Di Marzo, 2010). CB1 receptors are highly expressed in several brain regions and in lower densities outside the brain (Matsuda *et al*, 1990; Munro *et al*, 1993). In contrast, CB2 receptors have a more restricted distribution and are found mainly on immune cells and in low numbers in the brainstem (Van Sickle *et al*, 2005) and some other brain regions (Gong *et al*, 2006). Both CB1 and CB2 receptors primarily signal through inhibitory G proteins (Howlett *et al*, 2002).

Recent evidence indicates an important role for endocannabinoids and CB1 receptor activation in enhancing the memory consolidation of emotionally arousing experiences (Campolongo *et al*, 2009b; Graham *et al*, 2009). Moreover, it recently has been shown that the fatty acid amide hydrolase inhibitor URB597 enhances memory acquisition and consolidation in rats (Mazzola *et al*, 2009). These findings suggest that propofol might modulate memory consolidation of emotionally arousing experiences *via* an interaction with the endocannabinoid system. To investigate this issue, in a first experiment, anesthetic doses of propofol were administered to rats by intraperitoneal injection, immediately and 30, 90, and 180 min after aversively motivated inhibitory avoidance training, a widely used animal model to assess drug effects on emotional memory consolidation. In a second experiment, we evaluated whether the propofol effect on the consolidation of inhibitory avoidance memory is specific for this anesthetic by administering anesthetic doses of the benzodiazepine midazolam or the barbiturate pentobarbital immediately after inhibitory avoidance training. In the last experiment, we investigated whether the memory-enhancing effect of propofol depends on concurrent CB1 activity by administering a nonimpairing

dose of the CB1 receptor antagonist rimonabant 30 min before propofol injection; we also studied whether propofol administration modulates endocannabinoid release in rats.

Materials and Methods

Animals

Male adult Sprague-Dawley rats (350–450 g at the time of training; Charles River Laboratories, Calco, Italy) were housed individually and maintained in a temperature-controlled environment (20° ± 1°C) under a 24-h light-dark cycle (7:00 AM to 7:00 PM lights on) with unlimited access to food and water. All procedures involving animal care or treatments were approved by the Italian Ministry of Health (Rome, Italy) and performed in compliance with the guidelines of the US National Institutes of Health and the Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004).

Drug treatment

2,6-Diisopropyl phenol (propofol, 250, 300, or 350 mg/kg), purchased from Sigma-Aldrich (Milan, Italy), was dissolved in a vehicle containing 100% sesame oil. Midazolam (30, 50, or 70 mg/kg; Ratiopharm, Ulm, Germany) was dissolved in saline, and pentobarbital (60, 70, or 80 mg/kg; Sigma-Aldrich, St. Louis, MO) was dissolved in a vehicle containing 40% propylene glycol (1,2-propanediol), 10% ethanol, and 50% distilled water. Drug solutions were freshly prepared before each experiment and administered by intraperitoneal injection in a volume of 1 ml/kg immediately after the training trial. To control for time specificity, propofol was administered to different groups of rats either 30, 90, or 180 min after the training trial. To assess whether CB1 receptors are involved in mediating the propofol effect on memory consolidation, the CB1 receptor antagonist rimonabant (1 mg/kg; donated by the National Institute of Mental Health, Chemical Synthesis and Drug Supply Program, Bethesda, MD) was dissolved in a vehicle containing 5% polyethylene glycol, 5% TWEEN 80, and 90% saline (Bortolato *et al*, 2006) and administered immediately after training, whereas propofol was given 30 min later.

Behavioral studies

Inhibitory Avoidance Apparatus and Procedures. Rats were trained and tested in an inhibitory avoidance apparatus consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor. The starting compartment (31 cm long) was made of opaque white plastic and illuminated by a lamp; the shock compartment (60 cm long) was made of two dark, electrifiable metal plates and was not illuminated (McGaugh *et al*, 1988). Training and testing were performed during the light phase, between 10:00 AM and 2:00 PM, and were conducted in dim light conditions in a sound-attenuated room. Animals were handled 1 min each for 2 days before the training day.

For training, the rats were placed into the starting compartment of the apparatus, facing away from the door, and were permitted to explore the apparatus. After the rats stepped completely into the dark compartment, the sliding door was closed and a single, inescapable foot shock (0.35 mA) was delivered for 1 s. The animals were removed from the shock compartment 15 s after termination of the foot shock. Retention was tested 48 h later. On the retention test trial, the rats were placed into the starting compartment, and the latency to reenter the shock compartment with all four paws (maximum latency of 600 s) was recorded and used as a measure of retention. Longer latencies were interpreted as indicating better retention (Dawson and McGaugh, 1971). Immediately after the training and testing of each animal, the apparatus was cleaned with a 70% ethanol solution.

To be included in the test phase, rats they had to reach a minimum criterion on the training test (before treatment), which is 60 s maximum to step in the dark compartment of the maze.

All the analyses were performed by the same observer, who was unaware of animal treatment.

Sleeping Time. Sleeping parameters were determined in different groups of rats. To determine sleeping onset and recovery, immediately after anesthetic administration each rat was placed on its back once every 30 s until it was unable to right itself within 30 s. Sleeping onset was defined as the interval between anesthetic injection and the time the rat was unable to turn itself upright at least twice within 1 min. Then each rat was left undisturbed on its back until it

spontaneously regained its righting reflexes, defined as having at least three paws under its body. Complete recovery of the righting reflex was defined as the rat being able to turn itself upright. The time between loss and recovery of righting reflex for each rat was defined as sleeping time (cutoff=180 min) (Cagiano *et al*, 2002). All of the analyses were performed by the same observer, who was unaware of animal treatment.

Endocannabinoid Measurement

In accordance with Patel's protocol in mice (Patel *et al*, 2003), rats were treated with propofol (300 mg/kg, intraperitoneally) or with its vehicle and killed 8 or 40 min after administration.

Brain and plasma samples were subjected to a lipid extraction process, and the endocannabinoid content of the lipid extracts was determined using isotope-dilution liquid chromatography-mass spectrometry as described previously (Schelling *et al*, 2006). The brain tissue was collected and stored at -80°C. Before the extraction process, tissues were weighted and homogenized in polypropylene tubes (Sarstedt, Numbrecht, Germany) and kept in ice water. Five hundred µl of the described homogenized tissue solution was transferred to a 2-ml Eppendorf tube, and 20 µl of internal standard and 1 ml methyl tertiary butyl ether (Sigma-Aldrich, Italy) were added to extract the endocannabinoids. The mixture was vortexed for 1 min and centrifuged at 12,000*g* for 6 min. The clear supernatant was transferred into a clean 5-ml polypropylene tube (Sarstedt) and evaporated under vacuum at 37°C. The residue of all evaporated samples was reconstituted in 100 µl acetonitrile, vortexed for 30 s, and sonicated in 4°C water for 15 min. A 20-µl aliquot of the clear solution was used for liquid chromatography-tandem mass spectrometry analysis. All samples were injected in duplicates.

Statistical Analysis

The training and retention latencies of rats were analyzed with one-way ANOVA. Time-dependent effects of propofol, the interactions between propofol and rimonabant, and propofol effects on endocannabinoid concentrations were analyzed with two-way ANOVAs. The source of the detected significances was determined by Tukey–Kramer *post hoc* tests. To determine whether learning had occurred, paired *t* tests were used to compare the training and retention latencies of the vehicle groups. Sleeping parameters were analyzed with Kruskal-Wallis one-

way ANOVA on ranks or Mann–Whitney U test because of their nonnormal distribution. StatView software (SAS Institute, Cary, NC) was used to conduct statistical analyses. Normal data are expressed as mean ± SD; nonparametric data are expressed as median and percentiles. Two-tailed testing was used for all the analyses. *P* values of < 0.05 were considered statistically significant. The number of rats per group is indicated in the figures and tables.

Results

Posttraining administration of propofol enhances 48-h inhibitory avoidance retention performance

This experiment examined whether immediate posttraining administration of propofol would enhance 48-h retention performance of inhibitory avoidance training. Average step-through latencies for all groups during training (*i.e.*, before foot shock and drug treatment) were 17.6 ± 13.7 s (mean ± SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,46}$ = 0.93, *P* = 0.43). The 48-h retention latencies of rats given vehicle immediately after training were significantly longer than their entrance latencies during the

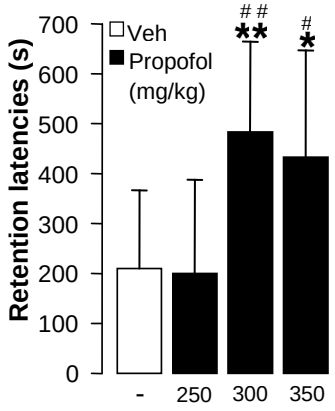

Fig. 1. Effects of posttraining administration of propofol on retention of an inhibitory avoidance response. Step-through latencies (mean ± SD) on a 48-h retention test. Immediate posttraining administration of propofol (300 mg/kg) enhanced memory retention. * *P* < 0.05; ** *P* < 0.01 *versus* vehicle; # *P* < 0.05; ## *P* < 0.01 *versus* 250 mg/kg propofol (n = 12, vehicle; n = 13, 250 and 300 mg/kg propofol; n = 9, 350 mg/kg propofol).

training trial (*t* = -5.59, *P* = 0.0002), indicating that the rats retained memory of the shock experience. As shown in figure 1, propofol induced dose-dependent retention enhancement. One-way ANOVA for 48-h retention latencies revealed a significant treatment effect ($F_{3,43}$ = 7.82, *P* = 0.0003). *Post hoc* analysis indicated that rats treated with the higher doses of propofol (300 or 350 mg/kg) had significantly longer retention than did those treated with vehicle or with 250

mg/kg propofol ($P < 0.01$ and $P < 0.05$ for 300 and 350 mg/kg, respectively). The lower dose of propofol (250 mg/kg), which did not induce anesthesia, did not induce retention enhancement. Three of 12 rats given 350 mg/kg propofol died of respiratory depression.

Propofol administered immediately or 30 min (but not 90 or 180 min) after the training enhanced 48-h inhibitory avoidance retention performance

To examine whether propofol influences the consolidation phase of memory processing, rats were treated with propofol (300 mg/kg) immediately or 30, 90, or 180 min after training. Average step-through latencies for all groups during training, before foot shock and drug treatment, were 16.6 ± 13.0 s (mean ± SD). Two-way ANOVA for training latencies revealed no significant differences between groups (main effect of treatment $F_{1,78} = 0.77$, $P = 0.38$; main effect of time of administration $F_{3,78} = 2.0$, $P = 0.12$; interaction $F_{3,78} = 1.54$, $P = 0.21$). Two-way ANOVA for 48-h retention latencies revealed a significant main effect of propofol ($F_{1,78} = 17.64$, $P < 0.0001$) as well as a significant main effect of time of administration ($F_{3,78} = 3.76$, $P = 0.014$). Moreover, there was a statistically significant interaction effect between treatment and time of administration ($F_{3,78} = 4.76$, $P = 0.0042$). As shown in figure 2, *post hoc* analysis indicated that rats treated with propofol either immediately or 30 min after training had significantly longer retention latencies than did those given vehicle ($P < 0.01$). Retention latencies of rats injected with propofol immediately or 30 min posttraining were significantly longer than were those of rats given propofol 180 min after the training ($P < 0.01$).

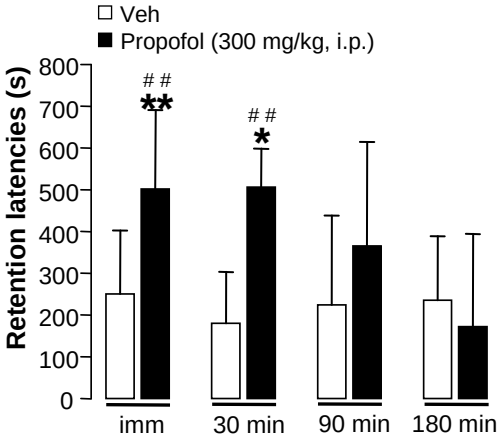

Fig. 2. Effects of immediate and delayed posttraining administration of propofol on retention of an inhibitory avoidance response. Step-through latencies (mean ± SD) on a 48-h retention test. Rats injected with propofol immediately or 30 min posttraining showed retention latencies longer than those of rats injected with vehicle at the corresponding time point and with propofol 180 min after training. ** $P < 0.01$ *versus* the corresponding vehicle group; ## $P < 0.01$ *versus* rats injected with propofol 180 min after training (n = 10, vehicle 30 min and 300 mg/kg propofol 90 min; n = 11, all other groups).

Posttraining administration of midazolam or pentobarbital does not enhance 48-h inhibitory avoidance retention performance

To determine whether the propofol effect on inhibitory avoidance memory enhancement is specific for this anesthetic, rats were treated with anesthetic doses of midazolam (30, 50, or 70 mg/kg, intraperitoneally) or pentobarbital (60, 70, or 80 mg/kg, intraperitoneally) immediately after inhibitory avoidance training. For midazolam, average stepthrough latencies for all groups during training, before foot shock and drug treatment, were 17.7 ± 13.9 s (mean ± SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,34}$ = 0.17, P = 0.92). As shown in figure 3A, one-way ANOVA for 48-h retention latencies indicated that midazolam did not significantly enhance retention latencies ($F_{3,34}$ = 0.09, P = 0.97). For pentobarbital, average step-through

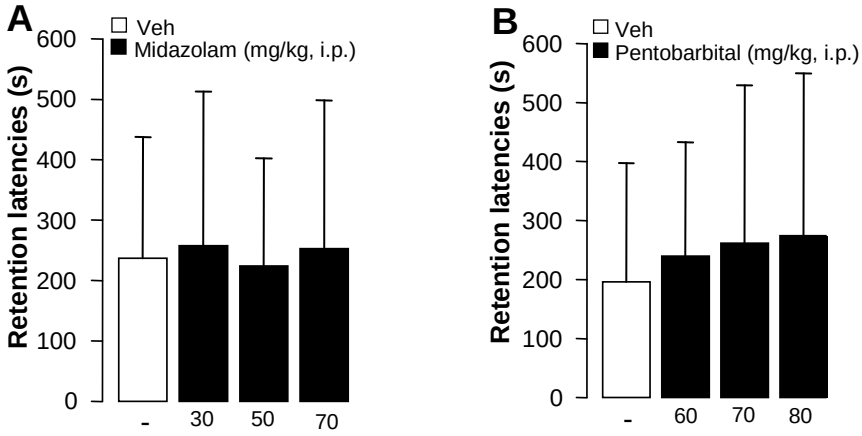

Fig. 3. Effects of posttraining administration of midazolam or pentobarbital on retention of an inhibitory avoidance response. Step-through latencies (mean ± SD) on a 48-h retention test. Immediate posttraining administration of midazolam (A) or pentobarbital (B) did not enhance memory consolidation (n = 9, 30 mg/kg midazolam and 70 or 80 mg/kg pentobarbital; n = 10 vehicle, 50 or 70 mg/kg midazolam and 60 mg/kg pentobarbital).

latencies for both groups during training, before foot shock and drug treatment, were 17.2 ± 14.2 s (mean ± SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,34}$ = 0.34, P = 0.79). As shown in figure 3B, one-way ANOVA for 48-h retention latencies indicated that pentobarbital did not significantly enhance retention latencies ($F_{3,34}$ = 0.21, P = 0.89).

The CB1 antagonist rimonabant blocks the memory enhancing effect induced by propofol

This experiment examined whether the memory-enhancing effect of propofol depends on a concurrent activation of CB1 receptors. To address this issue, we investigated whether the CB1 receptor antagonist rimonabant (1 mg/kg) administered intraperitoneally immediately after inhibitory avoidance training would block the retention enhancement induced by propofol given 30 min later. Average step-through latencies for all groups during training, before foot shock and drug treatment, were 15.2 ± 11.8 s. The 48-h retention latencies of rats given vehicle after training were significantly longer than their entrance latencies during the training trial ($P = 0.0001$). As shown in figure 4, posttraining administration of rimonabant blocked the retention enhancement induced by propofol (300 mg/kg). Two-way ANOVA for 8-h retention latencies revealed a significant rimonabant plus propofol interaction effect ($F_{1,27} = 11.70$, $P = 0.002$). *Post hoc* comparison revealed that retention latencies of rats given propofol alone were significantly longer than were those of vehicle-treated rats ($P < 0.01$). Most importantly, retention latencies of rats given an otherwise nonimpairing dose of rimonabant together with propofol were significantly shorter than those of rats treated with propofol alone ($P < 0.01$).

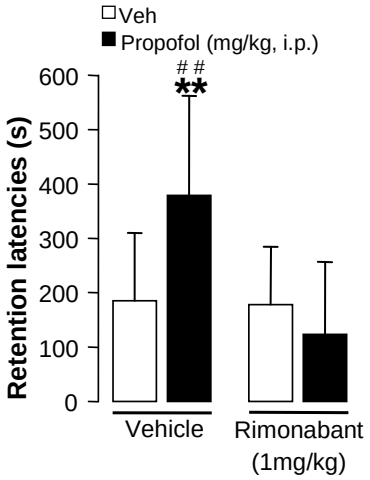

Fig. 4. Effects of the CB1 antagonist rimonabant on the memory-enhancing effects induced by propofol. Stepthrough latencies (mean ± SD) on a 48-h retention test. Immediate posttraining administration of the cannabinoid receptor antagonist rimonabant (1 mg/kg) blocked the memory-enhancing effects of propofol (300 mg/kg). ** $P < 0.01$ *versus* the corresponding vehicle group; ## $P < 0.01$ *versus* the corresponding propofol group (n = 7, 1 mg/kg rimonabant + vehicle propofol; n = 8, all other groups).

Sleeping time

Table 1 shows the effects of propofol, midazolam, and pentobarbital on sleeping parameters. Kruskal-Wallis ANOVA revealed no statistically significant effect on sleeping onset ($H_6 = 10.27$, $P = 0.11$).

However, Kruskal-Wallis ANOVA revealed a statistically significant effect for sleeping time ($H_6 = 19.64$, $P = 0.002$). *Post hoc* comparisons (Mann–Whitney U test with Bonferroni correction) revealed that rats given 50 mg/kg midazolam slept for a shorter amount of time than did rats given 70 or 80 mg/kg pentobarbital or those given 350 mg/kg propofol. None of the rats treated with the lower doses of midazolam (30 mg/kg) or propofol (250 mg/kg) lost righting reflex.

Table 2 shows the effects of rimonabant on propofol in inducing anesthesia. Mann–Whitney U test showed no difference between rats pretreated with rimonabant compared with rats pretreated with vehicle on sleeping onset or time induced by propofol (U = 5.0, $P = 0.11$; U = 11.000, $P = 0.75$, respectively), indicating that the anesthetic effect of propofol is independent from the indirect activation of the endocannabinoid system.

Table 1. Sleeping parameters of propofol-, midazolam-, and pentobarbital-treated rats

	Treatment, mg/kg						
	Prop 300 (n = 6)	Prop 350 (n = 5)	Mid 50 (n = 6)	Mid 70 (n = 6)	Pento 60 (n = 6)	Pento 70 (n = 5)	Pento 80 (n = 6)
Onset time(min)							
Median	12.0	20.0	11.0	10.5	4.0	4.0	14.0
25^{th} Percentile	6.3	9.3	6.0	9.0	3.0	3.0	9.0
75^{th} Percentile	13.8	20.3	24.0	15.0	13.0	7.5	24.0
Sleep time (min)							
Median	180	180	86.5*	113	113	144	165
25^{th} Percentile	31.8	180	50.0	97.0	92.0	135	150
75^{th} Percentile	180	180	102	158	163	180	180

Prop, propofol; Mid, midazolam; Pento, pentobarbital. * $P < 0.05$ *vs.* 350 mg/kg propofol and 70 or 80 mg/kg pentobarbital.

Table 2. Sleeping parameters of rats treated with propofol alone or together with rimonabant

Treatment	Onset Sleeping (min)			Sleep Time (min)		
	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Vehicle rimonabant + propofol (n = 5)	16.0	11.0	17.0	180	142	180
Rimonabant + propofol (n = 5)	11.0	9.0	13.5	180	134	180

Endocannabinoid measurement

Two-way ANOVA for propofol effects on endocannabinoid content revealed a statistically significant interaction between treatment and time of administration

Table 3. Endocannabinoid Concentrations

Treatment	Anandamide		2-Arachidonoylglycerol	
	Brain (ng/g Tissue)	Plasma (ng/ml)	Brain (µg/g Tissue)	Plasma (ng/ml)
Vehicle (8 min, n = 6)	13.8 ± 7.1	0.9 ± 0.3	28.4 ± 27.1	0.3 ± 0.1
Propofol (8 min, n = 6)	50.7 ± 38.8*	0.9 ± 0.2	29.1 ± 19.9	0.3 ± 0.1
Vehicle (40 min, n = 5)	33.7 ± 29.2	1.1 ± 0.5	45.9 ± 41.7	0.4 ± 0.2
Propofol (40 min, n = 6)	16.3 ± 6.5	1.1 ± 0.5	46.3 ± 36.3	0.4 ± 0.2

* $P < 0.05$ *versus* vehicle-treated rats (8 min).

($F_{1,19} = 7.1$, $P = 0.015$). *Post hoc* comparisons revealed that propofol increases anandamide concentrations in rat brains 8 min after administration ($P < 0.05$, table 3).

Discussion

The current findings indicate that propofol, at anesthetic doses, enhances memory consolidation of inhibitory avoidance training in rats when administered immediately after the training experience. This memory enhancement is blocked by coadministration of the CB1 cannabinoid receptor antagonist rimonabant, suggesting that the enhancing effect of propofol on memory consolidation depends on an indirect activation of CB1 receptors. In contrast, midazolam and pentobarbital, two anesthetics that do not increase endocannabinoid signaling

(Patel *et al*, 2003), did not enhance the consolidation of memory of inhibitory avoidance training.

The current findings may appear at odds with preclinical and clinical findings indicating that propofol induces amnesia. For example, Veselis *et al.* (Veselis *et al*, 2009) reported that propofol inhibits conscious memory processes in human subjects soon after memory encoding and that it impairs the acquisition or encoding of material into long-term memory. In addition, propofol has been reported to induce amnesia of training in rats on the same inhibitory avoidance task used in the current study (Alkire *et al*, 2001). However, a critical difference between these investigations and the current study is that in the human studies, memory function was assessed shortly after drug administration, whereas in the preclinical study, rats were given the drug before training. Therefore, acute pharmacologic effects could have influenced directly both the acquisition and retention of the training. In contrast, in our study the drug was administered *after* the training and was not present during the acquisition phase. Thus, the enhancing effects of propofol on retention performance in our study are likely mediated by specific influences on the consolidation of memory of the training experience (McGaugh and Roozendaal, 2009). The use of posttraining drug manipulation is a widely accepted method for effectively dissociating memory processes from secondary behavioral effects of nonassociative nature, such as those related to sensory sensitivity (Cahill *et al*, 2001). Because retention testing took place 48 h after training and drug treatment, these findings further exclude residual pharmacologic effects as having a direct influence on behavior during retention testing. Moreover, the effect of posttraining propofol administration on retention enhancement was time dependent: propofol administration immediately or 30 min after inhibitory avoidance training resulted in memory enhancement, whereas administration of propofol 90 or 180 min after training was ineffective. Together these findings provide evidence that propofol enhances time-dependent processes underlying the consolidation of memory for emotionally arousing experiences. The posttraining drug administration protocol used in the current article has a translational value to humans. Acute sedation or even the induction of anesthesia immediately *after* a traumatic experience (*e.g.*, in the consolidation phase of a traumatic memory) is a common clinical scenario in emergency medicine and in the ICU.

Our findings demonstrate that propofol is able to enhance memory consolidation when administered immediately after the exposure to a traumatic

event and that this effect on memory depends on an indirect activation of the endocannabinoid system. In accordance with the behavioral data, we also found that propofol administration increases anandamide concentrations in the rat brain 8 min after injection, whereas anandamide plasma concentrations remain unaffected. Our data are in accordance with preclinical and clinical evidence. Patel *et al.* (Patel *et al*, 2003) demonstrated increased concentrations of anandamide in the mouse brain after systemic administration of propofol in contrast to the administration of benzodiazepines, barbiturates, or volatile anesthetics; the effect of propofol on anandamide concentrations is mediated by an inhibition of fatty acid amide hydrolase, the major degradation enzyme of anandamide (Patel *et al*, 2003). In humans undergoing general anesthesia, plasma concentrations of the endocannabinoid anandamide remained unchanged during propofol anesthesia but were significantly reduced during anesthesia with volatile agents (Schelling *et al*, 2006; Weis *et al*, 2010).

The basolateral complex of the amygdala (BLA) appears to be a critical site for mediating drug effects on memory performance, including those of propofol. One study reported that permanent neurotoxic lesions of the BLA produced with *N*-methyl-D-aspartate blocked the amnestic effect of pretraining propofol administration of rats trained on an inhibitory avoidance task (Alkire *et al*, 2001). We recently have shown that the endocannabinoid system in the BLA is involved in the enhanced consolidation of inhibitory avoidance memory and that CB1 activity within the BLA is essential for mediating glucocorticoid effects on long-term memory (Campolongo *et al*, 2009a; Campolongo *et al*, 2009b). Based on these findings, a new model has emerged (Hill and McEwen, 2009; Hill *et al*, 2010). In this model, stress-induced glucocorticoids bind to membrane-bound receptors in the BLA that activate a G-protein signaling cascade that induces endocannabinoid synthesis. The ensuing release of endocannabinoid ligands could diffuse to local γ-aminobutyric acid–mediated (GABAergic) terminals and inhibit γ-aminobutyric acid release onto noradrenergic terminals in the BLA. The end result of this process is an increased norepinephrine release within the BLA and subsequently an enhancement of emotional memory consolidation. Many sedative and anamnestic effects of general anesthetics, including those of propofol, crucially depend on γ-aminobutyric acid release. The current findings demonstrate that the enhancing effects of propofol on memory consolidation depend on concomitant CB1 receptor activity, so we hypothesize that the anamnestic effects of propofol are mediated by an endocannabinoid-induced

inhibition of γ-aminobutyric acid release, resulting in a more pronounced memory consolidation during stressful conditions when glucocorticoid signaling is high (Roozendaal *et al*, 2006).

The pharmacokinetic properties of midazolam, pentobarbital, and propofol differ to a large extent, but all three drugs share the pharmacodynamic capability to potentiate γ-aminobutyric acid neurotransmission (Goodman and Gilman's, 2008). Our results showing that rats treated with midazolam (50 mg/kg) slept less than did rats treated with propofol (350 mg/kg) or pentobarbital (70 or 80 mg/kg) are in accordance with clinical evidence showing that midazolam has a shorter half-life than propofol and barbiturates (Goodman and Gilman's, 2008). However, neither rats treated with the higher dose of midazolam nor the ones treated with pentobarbital showed differences in the sleeping parameters compared with those treated with propofol. Although propofol enhances memory consolidation through an activation of the endocannabinoid system, the anesthetic effect of propofol does not depend on this activation. The CB1 receptor antagonist rimonabant blocks the propofol-enhancing effect on memory consolidation but does not influence propofol's effects on sleeping. On the whole, these data suggest that, unlike midazolam and pentobarbital, propofol induces selective effects on memory consolidation, which are linked to the activation of the endocannabinoid system and not related to the potentiation of GABAergic neurotransmission.

These findings, together with the results showing that midazolam and pentobarbital, at anesthetic doses, did not influence memory consolidation strongly corroborate the hypothesis that propofol's effects on memory consolidation are not attributable to a general nonspecific anesthetic effect.

In summary, our study demonstrates that propofol enhances memory consolidation *via* an endocannabinoid-mediated mechanism. These effects are markedly different from those of other direct GABAergic agents such as midazolam or pentobarbital. These findings from animal experiments suggest that propofol should be used with caution in individuals during the aftermath of an acute traumatic event and may help to explain the increased incidence of aversive memories from intraoperative awareness seen in patients undergoing total intravenous anesthesia with propofol (Errando *et al*, 2008). Likewise, the findings suggest that pharmacologic manipulation of endocannabinoid signaling could be a useful intervention aimed at blocking memory consolidation immediately after a traumatic event.

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CHAPTER 3

NOVELTY-INDUCED EMOTIONAL AROUSAL MODULATES CANNABINOID EFFECTS ON RECOGNITION MEMORY AND ADRENOCORTICAL ACTIVITY

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Abstract

Although it is well established that cannabinoid drugs can influence cognitive performance, the findings describing both enhancing and impairing effects have been ambiguous. Here, we investigated the effects of posttraining systemic administration of the synthetic cannabinoid agonist WIN55,212-2 (0.1, 0.3, or 1.0 mg/kg) on short- and long-term retention of object recognition memory under two conditions that differed in their training-associated arousal level. In male Sprague-Dawley rats that were not previously habituated to the experimental context, WIN55,212-2 administered immediately after a 3-min training trial, biphasically impaired retention performance at a 1-h interval. In contrast, WIN55,212-2 enhanced 1-h retention of rats that had received extensive prior habituation to the experimental context. Interestingly, immediate posttraining administration of WIN55,212-2 to non-habituated rats, in doses that impaired 1-h retention, enhanced object recognition performance at a 24-h interval. Posttraining WIN55,212-2 administration to habituated rats did not significantly affect 24-h retention. In light of intimate interactions between cannabinoids and the hypothalamic–pituitary–adrenal axis, we further investigated whether cannabinoid administration might differently influence training-induced glucocorticoid activity in rats in these two habituation conditions. WIN55,212-2 administered after object recognition training elevated plasma corticosterone levels in non-habituated rats whereas it decreased corticosterone levels in habituated rats. Most importantly, following pretreatment with the corticosterone-synthesis inhibitor metyrapone, WIN55,212-2 effects on 1- and 24-h retention of non-habituated rats became similar to those seen in the low-aroused habituated animals, indicating that cannabinoid-induced regulation of adrenocortical activity contributes to the environmentally sensitive effects of systemically administered cannabinoids on short- and long-term retention of object recognition memory.

Introduction

Extensive evidence indicates that cannabinoids, either administered exogenously or released from endogenous sites, have pronounced effects on learning and memory (Marsicano and Lafenetre, 2009; Wilson and Nicoll, 2001). The cannabinoid system consists of two types of G-proteincoupled receptors (CB1 and CB2 receptors), endogenous ligands, and enzymes involved in their synthesis and inactivation (Piomelli, 2003). Endogenous ligands for cannabinoid receptors, that is, endocannabinoids, are synthesized on demand in an activity-dependent manner and released from postsynaptic neurons. They travel backward across the synapse to activate presynaptic CB1 receptors and modulate presynaptic functions (Piomelli, 2003). Cannabinoid signaling is crucial for certain forms of short- and long-term plasticity at both excitatory and inhibitory synapses (Deadwyler *et al*, 2007) and thereby contributes to various aspects of brain function, including learning and memory (Marsicano *et al*, 2009). However, evidence indicates that the mnemonic consequence of cannabis exposure in humans does not always correspond to the effects observed in laboratory animals administered with cannabinoid compounds systemically or into discrete brain regions. For example, although it is well appreciated that cannabis use can impair short-term memory and executive function in humans (Pattij *et al*, 2008), not all preclinical studies, using WIN55,212-2 or other synthetic cannabinoid agonists, were able to confirm these findings (Baek *et al*, 2009; Clarke *et al*, 2008; Kosiorek *et al*, 2003; Schneider *et al*, 2008; Suenaga and Ichitani, 2008). Cannabinoid effects on long-term memory in humans did not receive much attention and findings of preclinical studies are not unequivocal, independent of the pharmacodynamic properties of the drug used. Whereas some animal studies indicate that systemic or intracranial administration of cannabinoid agonists impairs the encoding and consolidation of long-term memory processing (Barros *et al*, 2004; Robinson *et al*, 2008), enhancing effects are reported as well (De Oliveira Alvares *et al*, 2008). Although such discrepancies are not unusual in memory research, the factors contributing to these conflicting findings are yet poorly understood.

Emerging evidence indicates that cannabinoid drugs can induce distinct and even opposite effects on anxiety and several other behaviors, depending on the aversiveness of the environmental context (Campolongo *et al*, 2012; Carlin *et al*, 1972; Haller *et al*, 2009; Szuster *et al*, 1988; Zanettini *et al*, 2011). Based on these

findings, we hypothesized that the level of emotional arousal that is associated with a training experience might also be a primary factor in determining the outcome of cannabinoid administration on learning and memory. Therefore, in the present study we investigated the effects of cannabinoid administration on both short- and long-term object recognition memory under two experimental conditions that differed with respect to their training-associated arousal level. By employing a previously described procedure (Okuda *et al*, 2004), one group of rats was not habituated to the training context, whereas the other group was extensively habituated to the experimental apparatus to decrease its novelty-induced stress response during the training trial. The cannabinoid receptor agonist WIN55,212-2 was administered intraperitoneally to either extensively habituated or not previously habituated rats immediately after the object recognition training trial. In the first experiment, retention was tested 1 h after the training trial to assess possible cannabinoid effects on short-term cognitive performance. In the second experiment, we investigated cannabinoid effects on long-term object recognition memory by assessing retention 24 h after the training trial and drug treatment.

In addition to direct actions in the brain, cannabinoids are crucially involved in regulating hypothalamic–pituitary–adrenocortical (HPA) axis activity (Atsak *et al*, 2012b; Campolongo *et al*, 2009b; Di *et al*, 2003; Hill *et al*, 2010b), shaping the corticosterone response to stressful stimulation. As it is well established that glucocorticoids hormones are potent modulators of learning and memory (Campolongo and Roozendaal, 2011; Okuda *et al*, 2004; Roozendaal, 2002; Roozendaal *et al*, 2006b; Schwabe *et al*, 2012), we next investigated whether the neuroendocrine consequence of cannabinoid administration on plasma corticosterone levels contributes to the environmentally sensitive effects of systemically administered WIN55,212-2 on object recognition memory. We first investigated whether WIN55,212-2 administration after object recognition training differentially affected the release of endogenous corticosterone in rats in the two habituation conditions. Further, to assess whether this cannabinoid-induced shaping of the corticosterone response plays a role in regulating the memory modulatory influence of WIN55,212-2, we examined whether pharmacological suppression of corticosterone synthesis with metyrapone altered the effects of WIN55,212-2 administration on both short- and long-term retention of object recognition memory.

Materials and methods

Animals. Male adult Sprague–Dawley rats (350–450 g at the time of training, Charles River Laboratories, Italy) were kept individually in an air-conditioned colony room (temperature: 21 ± 1 °C; relative humidity: 60 ± 10%; lights on from 0700 to 1900 hours). Pellet food and water were available ad libitum. Training and testing were performed during the light phase of the cycle between 1000 and 1400 hours. All procedures involving animal care and treatments were in accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92) and the European Communities Council Directive of 24 November 1986 (86/ 609/EEC).

Drug Treatment. The cannabinoid receptor agonist WIN55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl] pyrolol [1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate; 0.1, 0.3, and 1.0 mg/kg; Sigma-Aldrich) was administered intraperitoneally in a volume of 1.0 ml/kg immediately after the training trial. Prior findings indicate that WIN55,212-2, after intraperitoneal administration, crosses the blood–brain barrier (Saghafi *et al*, 2011). For adrenocortical suppression, the 11β-hydroxylase inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; 35 mg/kg; Sigma-Aldrich) was injected in a volume of 2.0 ml/kg 40 min before the training trial. Metyrapone is a selective inhibitor of glucocorticoid synthesis in animals and humans (Schimmer and Parker, 2001). It blocks the conversion of the corticosterone precursor deoxycorticosterone in the adrenal cortex, thereby preventing the release of endogenous corticosterone into the bloodstream (Strashimirov and Bohus, 1966). All drugs were dissolved in 5% polyethylene glycol, 5% Tween-80, and 90% saline. The vehicle solution contained 5% polyethylene glycol and 5% Tween-80 in saline only.

Object Recognition Task. The experimental apparatus was a gray open-field box (in cm, 40 wide x 40 deep x 40 high) with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). All rats were handled twice per day for 1 min each for 7 days preceding the training day. The rats were divided into two groups. One group of rats was not habituated to the experimental apparatus (WITHOUT-habituation condition), whereas the other group was extensively habituated to the experimental context (WITH-habituation condition) to decrease

their novelty stress to the apparatus during the training trial. During habituation, the rats were allowed to explore the apparatus in the absence of objects twice per day for 3 min each for 7 days (Okuda *et al*, 2004). On the training trial, each rat was individually placed in the experimental apparatus at the opposite end from the objects. The rat was allowed to explore two identical objects (A1 and A2) for 3 min, then was removed from the apparatus and, after drug treatment, returned to its home cage. To avoid the presence of olfactory trails, sawdust was stirred and the objects were cleaned with 70% ethanol after each trial. Rat's behavior was recorded by using a video camera positioned above the experimental apparatus. Exploration of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around or sitting on an object was not considered as exploration. The time spent exploring the two objects was taken as a measure of object exploration, and exploratory behavior of the experimental apparatus was analyzed by the total number of rearings and crossings. For crossings, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between squares was determined. Retention was tested either 1 or 24 h after the training trial. On the retention test trial, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial. All combinations and locations of objects were used to reduce potential biases due to preference for particular locations or objects. Each rat was placed in the apparatus for 3 min, and its behavior was recorded. Videos were analyzed by a trained observer who was unaware of treatment condition. The time spent exploring each object and the total time spent exploring both objects were recorded. To analyze cognitive performance, a discrimination index was calculated as the difference in time exploring the novel and the familiar object, expressed as the percentage ratio of the total time spent exploring both objects.

Plasma Corticosterone Levels. Corticosterone levels were determined in parallel groups of rats in the WITHOUT-habituation and WITH-habituation condition and in rats that were handled (twice per day for 7 days) but not trained. For the last experiment, the corticosterone-synthesis inhibitor metyrapone was injected 40 min prior to the training trial. As novelty stimulation triggers an HPA-axis response that leads to a corticosterone plasma peak at 15–30 min and returns to baseline by 60–90 min (Grota *et al*, 1997), rats were killed 30 min after training and WIN55,212-2 administration. Trunk blood was collected after decapitation and

samples were centrifuged at 1900 g for 20 min at 4 1C. Plasma was stored at -80 °C and analyzed for corticosterone using ELISA kits (Assay designs, Ann Arbor, MI, USA; IDS, Boldon, Tyne and Wear, UK) according to the manufacturer's instructions.

Statistics. All data are expressed as mean ± SEM. Data were analyzed by one- or two-way ANOVA, followed by Tukey's *post hoc* comparison tests or paired or unpaired Student's *t*-tests, when appropriate. One-sample *t*-tests were used to determine whether the discrimination index was different from zero. A probability level of o0.05 was accepted as statistically significant. Fourteen rats were removed from statistical analyses because they showed a total exploration time of o10 s on either training or testing. Prior findings indicate that such rats do not adequately acquire the task (Okuda et al, 2004).

Results

Posttraining WIN55,212-2 induces opposite effects on 1-h retention of object recognition memory of rats in the WITHOUT-habituation and WITH-habituation condition

This experiment investigated whether immediate posttraining injection of the cannabinoid receptor agonist WIN55,212-2 altered short-term performance on an object recognition task and whether this WIN55,212-2 effect was influenced by prior habituation to the experimental context.

Training trial. Two-way ANOVA for total exploration time of the two identical objects on the training trial revealed a significant habituation condition effect ($F_{1,88}$ = 11.46, P = 0.001), but no differences between posttraining drug groups or an interaction between habituation condition and later drug treatment. Rats in the WITHOUT-habituation condition showed significantly less total exploration of the two objects than rats in the WITH-habituation condition (t_{94} = -3.37, P = 0.001; Figure 1a). In contrast, examination of rats' exploratory behavior of the training apparatus during the training trial indicated that the rats in the WITHOUT-habituation condition explored the experimental apparatus more than the rats in the WITH-habituation condition. Figure 1b and c shows that the number of crossings and rearings were significantly higher in rats in the WITHOUT-habituation condition than that in rats in the WITH-habituation condition (t_{94} = 4.33, P < 0.0001 for crossings, t_{94} = 3.36, P = 0.001 for rearings).

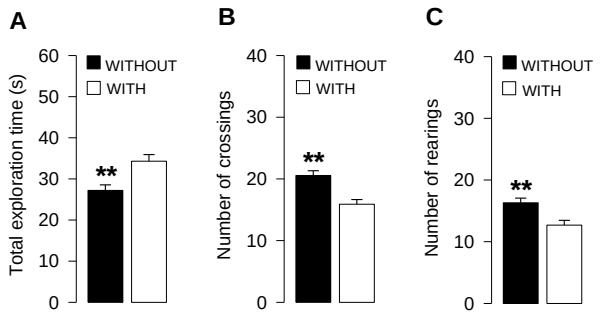

Figure 1. Behavioral effects during object recognition training in rats in the WITHOUT-habituation and WITH-habituation conditions. Rats in the WITHOUT-habituation (WITHOUT) condition spent significantly less time exploring the two identical objects during the training trial as compared with rats in the WITH-habituation (WITH) condition (A). Conversely, rats in the WITHOUT-habituation condition showed a higher number of crossings (B) and rearings (C) during training as compared with rats in the WITH-habituation condition. $^{**}P < 0.01$ vs the WITH-habituation group. Data are expressed as mean ± SEM (n = 48 per group).

Retention trial. One-sample *t*-tests revealed that the discrimination index of vehicle-treated rats was significantly different from zero in both the WITHOUT-habituation ($t_{11} = 4.27$, $P = 0.001$) and WITH-habituation condition ($t_{11} = 2.15$, $P = 0.05$), indicating that rats in both conditions discriminated the novel object at the 1-h retention interval. As shown in Figure 2, WIN55,212-2 administered immediately after the 3-min training trial, induced opposite effects on 1-h retention

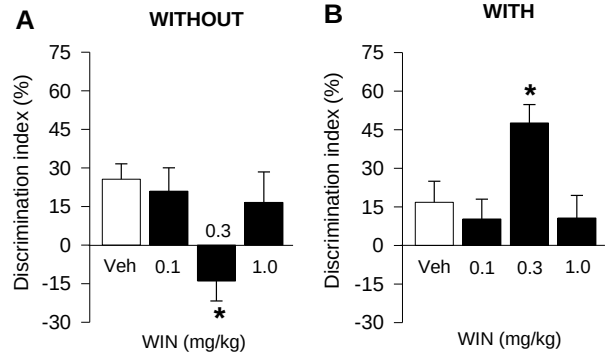

Figure 2. Effect of posttraining administration of WIN55,212-2 on 1-h retention of rats in the WITHOUT- and WITH-habituation conditions. Posttraining administration of WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) impaired 1-h retention of object recognition memory of rats in the WITHOUT habituation condition (A) but enhanced 1-h retention of rats in the WITH habituation condition (B). $^{*}P < 0.05$ vs the corresponding vehicle control group. Data are expressed as mean ± SEM (n = 12 per group).

performance of rats in the WITHOUT habituation and WITH-habituation conditions. Two-way ANOVA for discrimination index revealed no main effect of either drug treatment or habituation condition, but revealed a statistically significant interaction between these two factors ($F_{3,88} = 8.60$, $P < 0.0001$). *Post hoc* analysis indicated that the 0.3 mg/kg dose of WIN55,212-2, but not lower or higher doses, significantly decreased the discrimination index of rats in the WITHOUT-habituation condition ($P < 0.05$; Figure 2a). In contrast, the same dose of WIN55,212-2 increased the discrimination index of rats in the WITH-habituation condition ($P < 0.05$; Figure 2b). Two-way ANOVA for total exploration time of the two objects during the retention trial revealed no statistically significant effects of habituation condition, drug treatment or of the interaction between these two factors. Rats' exploratory behavior of the apparatus during the test trial also did not differ between rats in the WITHOUT-habituation and WITH-habituation condition (Table 1).

Table 1. Exploratory behavior of rats in the WITHOUT- and WITH-habituation conditions at the 1-h retention test

	Total object exploration time (s)	Number of crossings	Number of rearings
WITHOUT			
Vehicle	28.2 ± 3.3	14.0 ± 1.3	17.5 ± 2.2
WIN 0.1	21.3 ± 2.0	13.5 ± 1.2	15.5 ± 2.0
WIN 0.3	23.2 ± 1.8	11.0 ± 1.5	15.9 ± 3.2
WIN 1.0	22.8 ± 3.7	13.4 ± 2.4	14.9 ± 3.0
WITH			
Vehicle	25.9 ± 3.3	15.0 ± 1.9	15.9 ± 1.5
WIN 0.1	25.2 ± 3.3	14.6 ± 1.5	19.9 ± 3.2
WIN 0.3	30.8 ± 4.0	14.3 ± 1.7	15.3 ± 1.7
WIN 1.0	30.9 ± 3.9	12.6 ± 1.5	15.1 ± 2.2

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups in the WITHOUT- and WITH-habituation conditions. Results are expressed as mean±SEM (n = 12 per group).

Posttraining WIN55,212-2 enhances 24-h retention of object recognition memory of rats in the WITHOUT-habituation but not in the WITH-habituation condition

This experiment examined, in separate groups of rats, whether immediate posttraining injection of WIN55,212-2 influenced long-term performance on an

object recognition task and whether this WIN55,212-2 effect was also influenced by prior habituation to the experimental context.

Training trial. The pattern of effects on the training trial was highly comparable to that observed in the first experiment. Two-way ANOVA for total exploration time of the two objects on the training trial revealed a significant habituation condition effect ($F_{1,88}$ = 4.04, P = 0.05), but no differences between posttraining drug groups or an interaction between habituation condition and posttraining drug treatment. Rats in the WITHOUT-habituation condition explored the two objects significantly less (23.7 ± 1.3 s) than did rats in the WITH-habituation condition (28.2 ± 1.9 s) (t_{94} = -2.05, P < 0.05). Also, as found in the first experiment, rats in the WITHOUT-habituation condition showed significantly more exploration of the experimental apparatus than did rats in the WITH-habituation condition, as indicated by a higher number of crossings (t_{94} = 2.89, P = 0.005) and rearings (t_{94} = 2.92, P = 0.004).

Retention trial. As expected, after a 3-min training trial, rats of both vehicle groups did not express long-term retention of the familiar object: One-sample *t*-tests revealed no preference for the novel object in vehicle-treated rats in either the WITHOUT-habituation or WITH-habituation condition. As shown in Figure 3, posttraining WIN55,212-2 induced different effects on object recognition memory of rats in the WITHOUT-habituation and WITH-habituation groups at the 24-h retention interval. Two-way ANOVA for discrimination index indicated no main

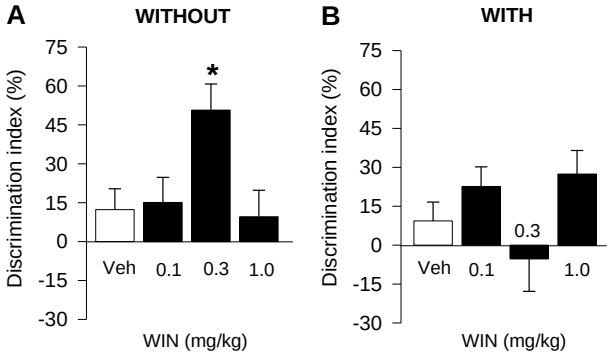

Figure 3. Effect of posttraining administration of WIN55,212-2 on 24-h retention of rats in the WITHOUT- and WITH-habituation conditions. Posttraining administration of WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) enhanced 24-h retention of object recognition memory of rats in the WITHOUT-habituation condition (A) and did not significantly affect 24-h retention of rats in the WITH-

habituation condition (B). *P < 0.05 vs the corresponding vehicle control group. Data are expressed as mean ± SEM (n = 10–14 per group).

effect of either drug treatment or habituation condition, but revealed a statistically significant drug treatment x habituation condition interaction ($F_{3,88}$ = 5.65, P = 0.001). WIN55,212-2 improved 24-h retention of rats in the WITHOUT-habituation condition in an inverted U-shape relationship ($F_{3,49}$ = 3.81, P = 0.02; Figure 3a), without significantly affecting 24-h retention of rats in the WITH-habituation condition ($F_{3,39}$ = 2.49, P = 0.07 Figure 3b). Post hoc analysis indicated that the 0.3 mg/kg dose of WIN55,212-2, but not lower or higher doses, administered to rats in the WITHOUT-habituation condition increased the discrimination index as compared with vehicle controls (P < 0.05). Two-way ANOVA for total exploration time of the two objects during the retention trial revealed a significant habituation condition effect ($F_{1,88}$ = 6.67, P = 0.01), but no drug treatment effect or interaction between both factors (Table 2). Comparable to the findings of the first experiment, rats' exploratory behavior of the experimental apparatus during the retention test trial did not differ between rats in the WITHOUT-habituation and the WITH-habituation conditions (Table 2).

Table 2. Exploratory behavior of rats in the WITHOUT- and WITH-habituation conditions at the 24-h retention test

	Total object exploration time (s)	Number of crossings	Number of rearings
WITHOUT			
Vehicle	18.0 ± 1.7	13.2 ± 1.4	12.3 ± 1.2
WIN 0.1	19.9 ± 2.1	13.4 ± 1.2	12.2 ± 0.9
WIN 0.3	15.5 ± 0.7	13.8 ± 1.6	14.3 ± 1.1
WIN 1.0	22.6 ± 2.3	10.5 ± 0.9	11.8 ± 1.2
WITH			
Vehicle	25.0 ± 3.8	13.9± 1.9	12.9 ± 1.2
WIN 0.1	18.8 ± 1.7	11.0 ± 1.1	11.1 ± 1.5
WIN 0.3	26.0 ± 3.9	12.9 ± 2.6	13.3 ± 2.6
WIN 1.0	24.1 ± 2.3	10.3 ± 1.1	12.3 ± 2.2

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups in the WITHOUT- and WITH-habituation conditions. Results are expressed as mean ± SEM (n = 10–14 per group). Results are expressed as mean ± SEM (n = 12 per group).

Posttraining WIN55,212-2 induces opposite effects on plasma corticosterone levels in rats in the WITHOUT-habituation and WITH-habituation condition

Figure 4 shows plasma corticosterone levels of parallel groups of trained rats in the WITHOUT-habituation or WITH-habituation condition, as assessed 30 min after

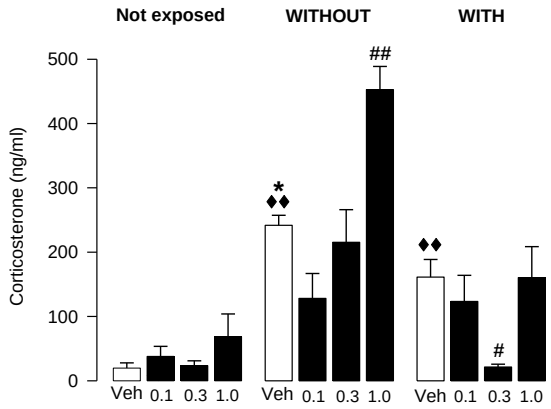

Figure 4. Effect of object recognition training and posttraining administration of WIN55,212-2 on plasma corticosterone levels in rats in the WITHOUT- and WITH-habituation conditions. Plasma corticosterone levels in rats in the WITHOUT-habituation condition treated with vehicle posttraining were significantly higher than those in vehicle-injected rats in the WITH-habituation condition, as assessed 30 min after training; **P* < 0.05 vs vehicle-treated rats in the WITH-habituation condition. Further, plasma corticosterone levels in vehicle-treated trained rats in both the WITHOUT- and WITH-habituation conditions were significantly higher than those in non-trained vehicle-treated rats; ♦♦*P* < 0.01 vs vehicle-treated not exposed rats. WIN55-212,2 (WIN, 1 mg/kg, i.p.) administered immediately after object recognition training increased plasma corticosterone levels in rats in the WITHOUT-habituation condition; ##*P* < 0.01 vs the corresponding vehicle-treated control group. In contrast, WIN55,212-2 (0.3 mg/kg, i.p.) decreased plasma corticosterone levels in habituated rats; #*P* < 0.05 vs the corresponding vehicle-treated control group. Data are expressed as mean ± SEM (n = 4–7 per group).

the training trial and WIN55,212-2 injection. Another group of rats was administered WIN55,212-2, but was only handled and not trained (home cage). Concerning plasma corticosterone levels in rats treated with vehicle only, one-way ANOVA revealed a significant habituation condition effect ($F_{2,14}$ = 30.75, *P* < 0.0001). *Post hoc* comparison tests showed that plasma corticosterone levels in rats in the WITHOUT-habituation condition treated with vehicle were significantly higher than those in vehicle-injected rats in the WITH-habituation condition (*P* < 0.05), supporting the view that the habituation procedure efficiently suppressed the level of emotional arousal during the training trial. Further, plasma corticosterone levels in vehicle-treated trained rats in both the WITHOUT- and WITH-habituation conditions were significantly higher than those in non-trained vehicle-treated rats (*P* < 0.01 for both comparisons).

WIN55,212-2 induced opposite effects on plasma corticosterone levels in rats after object recognition training, depending on the level of emotional arousal at the time of drug administration. Two-way ANOVA revealed a significant habituation condition effect ($F_{2,56} = 41.19$, $P < 0.0001$), drug treatment effect ($F_{3,56} = 9.58$, $P < 0.0001$) and interaction between both factors ($F_{6,56} = 4.78$, $P = 0.0005$). *Post hoc* analysis indicated that the 1.0 mg/kg dose of WIN55,212-2 elevated plasma corticosterone levels in rats in the WITHOUT-habituation condition ($P < 0.01$). In contrast, the 0.3 mg/kg dose of WIN55,212-2, but not any of the other doses, decreased plasma corticosterone levels when administered to rats in the WITH-habituation condition ($P < 0.05$). WIN55,212-2 administration did not significantly alter plasma corticosterone levels in non-trained control rats.

Adrenocortical suppression in rats in the without-habituation condition modifies the effect of posttraining WIN55,212-2 on short- and long-term object recognition memory

The findings described above indicate that WIN55,212-2 administered to rats in the two habituation conditions not only induces opposite effects on short- and long-term retention of object recognition memory but also on plasma corticosterone levels. To determine whether the WIN55,212-2 effect on the corticosterone response contributes to how WIN55,212-2 influences object recognition memory, in the last experiment we investigated whether pharmacological suppression of adrenocortical activity with metyrapone (35 mg/kg, i.p.), administered to non-habituated rats 40 min prior to the training trial, altered the effects of posttraining WIN55,212-2 administration on short- and long-term object recognition memory.

Training trial. Two-way ANOVA for total exploration time of the two identical objects on the training trial revealed no significant metyrapone effect, no difference between posttraining WIN55,212-2 treatment groups or interaction between these two parameters. Two-way ANOVAs for the number of crossings and rearings on the training trial also did not reveal any significant metyrapone or later WIN55,212-2 treatment effect.

One-hour retention. As shown in Figure 5a, pretreatment of rats in the WITHOUT-habituation condition with metyrapone transformed the effect of posttraining WIN55,212-2 administration on 1-h retention performance into that of rats in the WITH-habituation condition. Two-way ANOVA for discrimination index indicated no main effect of metyrapone or WIN55,212-2 treatment, but

revealed a significant metyrapone x WIN55,212-2 interaction effect ($F_{1,35}$ = 11.83, P = 0.02). Comparable to the findings shown in Figure 2a, WIN55,212-2 (0.3 mg/kg) administered alone to non-habituated rats immediately after the training

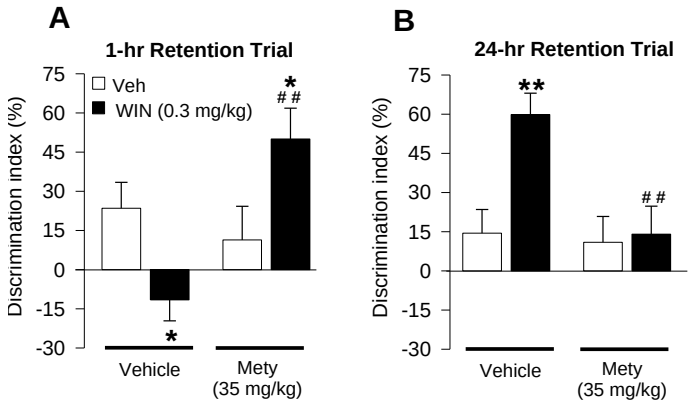

Figure 5. Effect of pretreatment with metyrapone on the effect of posttraining administration of WIN55,212-2 on 1- and 24-h retention of rats in the WITHOUT-habituation condition. Metyrapone (Mety, 35 mg/kg, i.p.) administered to rats in the WITHOUT-habituation condition 40 min before object recognition training reverted the impairing effect of posttraining WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) on 1-h retention (A) and the enhancing effect of WIN55,212-2 (0.3 mg/kg, i.p.) on 24-h retention (B). Both 1- and 24-h retention performance became very similar to those observed in low-aroused rats in the WITH-habituation condition (see Figures 2B and 3B). *P < 0.05; **P < 0.01 vs the corresponding vehicle group, ##P < 0.01 vs the WIN55,212-2 alone group. Data are expressed as mean ± SEM (n = 9–12 per group).

trial significantly decreased the discrimination index relative to that of vehicle-treated rats (P < 0.05). However, in non-habituated rats pretreated with metyrapone, the same dose of WIN55,212-2 significantly increased the discrimination index (P < 0.05 vs vehicle) and thus highly resembled the WIN55,212-2 effect on 1-h retention as described above for rats in the WITH-habituation condition (see, Figure 2b). Metyrapone pretreatment by itself did not significantly alter the discrimination index. Total exploration time of the two objects and rats' exploratory behavior of the apparatus on the 1-h retention test did not differ between drug treatment groups (Table 3).

Table 3. Exploratory behavior of rats in the WITHOUTH-habituation condition injected with metyrapone and WIN55,212-2 at the 1- and 24-h retention tests

Treatment		Total object exploration time (s)	Number of crossings	Number of rearings
1-h Retention trial				
Vehicle	Vehicle	24.9 ± 2.3	12.7 ± 1.5	15.9 ± 3.0
Vehicle	WIN 0.3	23.1 ±1.8	11.6 ± 1.5	14.9 ± 3.6
Mety 35	Vehicle	19.3 ± 2.2	11.9 ± 1.8	17.4 ± 2.8
Mety 35	WIN 0.3	21.4 ± 3.0	11.1 ±2.3	13.5 ± 3.0
24-h Retention trial				
Vehicle	Vehicle	21.3 ± 3.5	14.5 ± 1.5	8.3 ± 0.9
Vehicle	WIN 0.3	16.5 ± 0.8	15.5 ± 0.8	12.1 ± 1.2
Mety 35	Vehicle	25.4 ± 3.0	15.3 ± 2.3	12.1 ± 1.9
Mety 35	WIN 0.3	20.3 ± 3.1	14.2 ± 2.4	11.6 ± 2.5

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of rats in the WITHOUT-habituation condition administered metyrapone (Mety, mg/kg, i.p.) 40 min prior to training and WIN55,212-2 (WIN, mg/kg, i.p.) immediately posttraining. Results are expressed as mean ± SEM (n = 10–12 per group).

Twenty-four-hour retention. As shown in Figure 5b, pretreatment of non-habituated rats with metyrapone also transformed the effect of posttraining WIN55,212-2 on 24-h retention performance into that of rats in the WITH-habituation condition. Two-way ANOVA for discrimination index indicated no main effect of either metyrapone or WIN55,212-2 treatment, but revealed a significant metyrapone_WIN55,212-2 interaction effect ($F_{1,40}$ = 4.94, P = 0.03). Comparable to the findings shown in Figure 3a, WIN55,212-2 (0.3 mg/kg)

administration alone significantly increased the discrimination index of rats in the WITHOUT-habituation condition (P < 0.01). However, in rats pretreated with metyrapone, posttraining WIN55,212-2 administration did not significantly alter retention performance on the 24-h test trial, thus resembling the findings described above for rats in the WITH-habituation condition (see, Figure 3b). Metyrapone pretreatment alone did not significantly alter the discrimination index. Total exploration time of the two objects during the 24-h retention trial and rats' exploratory behavior of the apparatus did not differ between drug treatment groups (Table 3).

Plasma corticosterone levels. Table 4 shows the effect of metyrapone pretreatment and posttraining WIN55,212-2 administration on plasma corticosterone levels in rats in the WITHOUT-habituation condition, as assessed 30 min after object recognition training and WIN55,212-2 administration. Rats treated with metyrapone 40 min prior to the training trial had significantly lower

plasma corticosterone levels than rats administered vehicle before training (t_8 = 3.44, P = 0.009). In rats pretreated with metyrapone, posttraining WIN55,212-2 administration did not significantly elevate plasma corticosterone levels.

Table 4. Plasma corticosterone levels in rats in the WITHOUT-habituation condition injected with Metyrapone and WIN55,212-2 at the 1- and 24-h retention tests

Mety	WIN	Cort, ng/ml
Vehicle	Vehicle	245.3 ± 12.7
Mety 35	Vehicle	158.1 ± 22.0**
Mety 35	WIN 0.1	190.0 ± 47.8
Mety 35	WIN 0.3	222.8 ± 40.4
Mety 35	WIN 1.0	180.1 ± 20.3

Plasma corticosterone (Cort) levels (mean ± SEM) in ng/ml in rats in the WITHOUT-habituation condition as assessed 30 min after the training trial. Metyrapone (Mety, mg/kg, i.p.), injected 40 min prior to training, reduced training-induced plasma corticosterone levels and prevented the increase in corticosterone plasma levels induced by posttraining WIN55,212-2 (WIN, mg/kg, i.p.) administration. **P < 0.01 vs the vehicle control group (n = 4–5 per group).

Discussion

Using a previously described habituation procedure, we show that the synthetic cannabinoid agonist WIN55,212-2 interacts with training-associated emotional arousal in influencing both short- and long-term object recognition memory. Furthermore, our findings indicate that the cannabinoid system shapes the response of the HPA-axis to emotional arousal and that this neuroendocrine action is importantly involved in determining how WIN55,212-2 might affect object recognition memory. WIN55,212-2, administered systemically immediately after object recognition training, biphasically impaired retention at a 1-h interval in rats that were not habituated to the experimental context. In contrast, the same dose of WIN55,212-2 enhanced 1-h retention of rats that had reduced novelty-induced emotional arousal because of extensive prior habituation to the experimental context. Additionally, immediate posttraining administration of WIN55,212-2 to non-habituated rats, in a dose that impaired 1-h retention, enhanced object recognition performance at a 24-h interval. Posttraining WIN55,212-2 administration did not significantly affect 24-h retention of

habituated rats. This habituation procedure also produced opposite cannabinoid effects on training-induced HPA-axis activity. WIN55,212-2 administration after object recognition training elevated plasma corticosterone levels in rats that were not previously habituated to the experimental context, but decreased levels in habituated rats. Most importantly, as non-habituated rats administered the corticosterone-synthesis inhibitor metyrapone prior to training and WIN55,212-2 injection performed equally to extensively habituated rats on short- and long-term retention tests, these findings indicate that cannabinoid-induced regulation of adrenocortical activity contributes to the environmentally sensitive effects of systemically administered cannabinoids on short- and long-term retention of object recognition memory. Table 5 summarizes our findings of WIN55,212-2 on both short- and long-term recognition memory and on adrenocortical activity.

Table 5. Schematic representation of the effects of posttraining WIN55,212-2 on recognition memory

Habituation condition	Treatment	Short-term memory effects	Long-term memory effects
WITHOUT	WIN	impairing	enhancing
WITH	WIN	enhancing	---
WITHOUT	WIN + Mety	enhancing	---

Schematic representation of the effects of posttraining WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) either alone or together with the corticosterone-synthesis inhibitor metyrapone (Mety, 35 mg/kg, i.p.) on 1- and 24-h retention performance in rats in the WITHOUT-habituation and WITH-habituation conditions.

Clinical evidence indicates that cannabis use, either chronic consumption or acute intoxication, can impair both short-term memory and executive function (Pattij *et al*, 2008). Although some preclinical studies were able to confirm these findings (Baek *et al*, 2009; Kosiorek *et al*, 2003), the literature appears highly ambiguous (Clarke *et al*, 2008; Suenaga *et al*, 2008). In a previous study, we demonstrated that pretraining enhancement of endocannabinoid tone biphasically modified short-term retention of a recognition task in a spatial open field, and that this effect was likely influenced by the emotional state of the rat during the training trial (Campolongo *et al*, 2012). Here, we employed posttraining drug administration in order to more properly investigate the cognitive processes

involved and found that the level of emotional arousal at encoding influenced the outcome of later administration of the cannabinoid agonist WIN55,212-2 on both short- and long-term retention of object recognition memory. As vehicle-control rats in both habituation conditions showed a similar preference for the novel object at the 1-h retention interval, it is unlikely that any difference in acquisition (ie, total exploration of the objects during the training trial) between the two groups underlies the differential effects. It seems most likely that a difference in the arousal level induced by the habituation procedure was critical. The finding that non-habituated rats displayed significantly higher levels of locomotion and rearing behavior during the training trial than habituated rats is consistent with previous evidence that exposure of rats to novel contexts induces changes in behavioral responses, including hyperlocomotion and increased rearing behavior (Okuda *et al*, 2004; van den Buuse *et al*, 2001). Novelty-induced arousal also activates stress hormone systems, including glucocorticoids (Emmert and Herman, 1999; Handa *et al*, 1994) and epinephrine (Feenstra *et al*, 2000; McQuade *et al*, 1999). Corroborating previous findings by Okuda et al (2004), we found that non-habituated rats had a significantly more pronounced training-induced increase in plasma corticosterone levels than habituated rats. Although these behavioral and endocrine measures clearly indicate that our habituation procedure efficiently reduced the level of emotional arousal during the training session, we cannot exclude the possibility that it is the stress history and not the level of emotional arousal per se that may be influencing cannabinoid effects on memory. Repeated exposure to the testing arena might be mildly stressful to the animals. Prior evidence indicated that a history of repeated (restraint) stress reduces CB1 receptor function on GABAergic terminals within several brain regions involved in learning and memory (Hu *et al*, 2011; Patel *et al*, 2009). As such, the divergent effects of WIN55,212-2 on short-term retention of rats in the WITHOUT-habituation and WITH-habituation groups may be due to a different ability of CB1 receptor activation to suppress GABAergic inhibitory control because of the stress history of the animals and not the immediate levels of arousal during the training session. In both scenarios, however, our findings show that the cannabinoid system exerts an environmentally sensitive modulation of short-term memory. As WIN55,212-2 levels are probably still elevated at the time of the 1-h retention test, it is most likely that WIN55,212-2 affected short-term retention performance via direct influences on the retrieval of memory processing (Atsak *et al*, 2012a).

Whereas clinical studies mainly focused on the potentially disruptive effects of cannabinoid drugs on short-term cognitive performance, findings of animal experiments provide extensive evidence that cannabinoid treatments also influence long-term memory. However, findings of these experiments have been conflicting as well. Some studies reported that cannabinoid agonists impair the consolidation of long-term memory of training on several tasks (Barros *et al*, 2004; Robinson *et al*, 2008), whereas others found enhancing effects (De Oliveira Alvares *et al*, 2008). Differences in dosage or drug administration regimen (eg, pretraining vs posttraining administration) could have contributed to these opposite findings. Here we show that, similar to WIN55,212-2 effects on short-term memory, the level of emotional arousal at encoding, or alternatively the stress history of the animals, is another important factor modulating cannabinoid effect on long-term memory. WIN55,212-2 enhanced 24-h retention of rats that were not previously habituated to the experimental context, but failed to significantly alter performance of well-habituated rats. As WIN55,212-2 was administered immediately after the training trial, the effect of WIN55,212-2 on long-term retention is likely mediated by a selective influence on the consolidation of memory (McGaugh and Roozendaal, 2002). Moreover, because retention testing took place 24 h after the training and drug treatment, it is unlikely that the effects are mediated by any residual pharmacological effects on directly influencing behavior during retention testing (Cahill *et al*, 2001).

Clinical evidence supports the view that cannabis consumption can induce opposite effects on a variety of behaviors and subjective feelings in different individuals or even in the same subject (Green *et al*, 2003). Apart from genetic vulnerability (Xian *et al*, 2008), environmental factors such as stress-induced alterations could contribute to these differential effects (Viveros *et al*, 2012; Xian *et al*, 2008). Preclinical models have confirmed opposite effects of cannabinoid drugs on motor activity (Rodriguez de Fonseca *et al*, 1998), positive incentive and/or motivational processes (Chaperon and Thiebot, 1999), anxiety (Haller *et al*, 2009) and fear-related behaviors (Moreira and Wotjak, 2011). Although differences in dose, route of administration, and timing of exposure are typically listed as being responsible for the opposite behavioral effects, recent findings indicate that variations in the stressfulness of the experimental conditions employed in the different studies are implicated as well. The general assumption that enhancement of cannabinoid levels via an inhibition of the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH) results in anxiolytic- and

antidepressant-like effects in rodents (Gobbi *et al*, 2005; Kathuria *et al*, 2003) has recently been revisited. Haller et al (2009) found robust anxiolytic-like effects of the FAAH inhibitor URB597 when rats were tested without prior habituation to the experimental room. However, URB597 administration did not induce any anxiolytic-like effect in rats that were habituated to the testing environment. Similarly, both low and high doses of delta-9-tetrahydrocannabinol, the main psychoactive constituent of cannabis preparations, exerted anxiolytic-like effects in non-stressed animals (Fokos and Panagis, 2010). In stressed animals, however, only the higher dose of delta-9-tetrahydrocannabinol induced an anxiolytic-like response whereas the lower dose induced an anxiogenic effect. Our present findings are thus very reminiscent of these other behaviors and indicate that the level of emotional arousal that is associated with the training procedure is also a key regulator of cannabinoid effects on both short- and long-term recognition memory.

Considering the extensive evidence indicating a close relationship between the cannabinoid system and HPA-axis activity (Armario, 2010; Barna *et al*, 2004), we explored the possibility that the divergent effects of systemic WIN55,212-2 administration on object recognition memory might be related to differential effects of WIN55,212-2 on training-induced glucocorticoid levels in rats in these two habituation conditions. Our findings indicate that WIN55,212-2 administration produced opposite effects on training-induced plasma corticosterone levels in habituated vs non-habituated rats. Generally, these findings are thus consistent with the evidence that the cannabinoid system is an important regulator of HPA-axis activity and consequently of the release of glucocorticoid hormones (Cota *et al*, 2007; Ganon-Elazar and Akirav, 2009). Our finding that WIN55,212-2 induced opposite effects on HPA-axis activity in rats in the two habituation conditions has a twofold interest. First, these findings might help to reconcile earlier observations that cannabinoid administration can both activate and inhibit the HPA-axis (Cota *et al*, 2007; Ganon-Elazar *et al*, 2009). Second, as extensive evidence indicates that glucocorticoid hormones influence both short- and long-term memory of emotionally arousing experiences (Roozendaal *et al*, 2009; Schwabe *et al*, 2012), these findings suggest the intriguing possibility that this arousal-dependent influence of WIN55,212-2 on the HPAaxis might contribute to the observed opposite effects of WIN55,212-2 on both short- and long-term memory. Highly comparable to our present findings with WIN55,212-2, it has been previously reported that corticosterone

administration after object recognition training impaired short-term performance in emotionally aroused non-habituated rats (Okuda *et al*, 2004; Roozendaal *et al*, 2006b). Moreover, corticosterone enhanced 24-h retention performance of rats that were not previously habituated to the experimental context. Corticosterone administration to well-habituated rats failed to affect either short- or long-term object recognition performance. Even though the dose of WIN55,212-2 that affected memory function in the non-habituated rats did not overlap entirely with its efficacious dose on corticosterone levels, our assumption was confirmed by the finding that adrenocortical suppression with the corticosterone-synthesis inhibitor metyrapone in non-habituated rats altered the effect of posttraining WIN55,212-2 administration on both short- and long-term recognition memory in such a way that performance became similar to that seen in habituated animals. Thus, these findings provide strong support for the view that a WIN55,212-2-induced potentiation of glucocorticoid secretion plays an important role in determining the pattern of cannabinoid effects on memory in these animals.

Previously, we showed that the cannabinoid and glucocorticoid effects on memory might converge within the basolateral amygdala (BLA). The possible interaction of these two systems was originally investigated for memory consolidation of inhibitory avoidance training (Campolongo *et al*, 2009b) but similar mechanisms might exist in other brain regions for regulating other memory functions (Atsak *et al*, 2012a). Extensive evidence indicates that the BLA preferentially modulates memory of emotionally arousing training experiences (McGaugh, 2000) and that arousal-induced activation of noradrenergic signaling plays an important role herein (Quirarte *et al*, 1997; Roozendaal, 2002; Roozendaal *et al*, 2004; Roozendaal *et al*, 2006a). In prior studies, we showed that training-induced noradrenergic activity within the BLA is required for enabling the modulatory influence of glucocorticoids on memory (Quirarte *et al*, 1997). As habituation to the object recognition apparatus is known to attenuate training-induced increases in noradrenergic activity (Roozendaal *et al*, 2006b), we previously hypothesized that glucocorticoids might not modulate memory of object recognition training in habituated rats because of inadequate levels of arousal-induced norepinephrine. In support of this interpretation, we found that stimulating noradrenergic activity with the α_2-adrenoceptor antagonist yohimbine was sufficient to enable glucocorticoid-induced memory enhancement in habituated rats (Roozendaal *et al*, 2006b). Recent findings indicate that the endocannabinoid system within the BLA might be importantly involved in

regulating glucocorticoid effects on memory (Campolongo *et al*, 2009b). Systemically administered corticosterone rapidly elevates endocannabinoid levels in the amygdala (Hill *et al*, 2010a), whereas a blockade of CB1 receptor activity in the BLA prevents corticosterone-induced memory enhancement (Campolongo *et al*, 2009b). On the basis of these findings, we previously proposed a model in which glucocorticoids bind to a membrane-bound receptor in the BLA that activates a G-protein signaling cascade to stimulate the synthesis of endocannabinoids. The ensuing release of endocannabinoid ligands could diffuse to local GABAergic terminals and inhibit GABA release onto noradrenergic terminals in the BLA (Campolongo *et al*, 2009a; Hill and McEwen, 2009). Importantly, as recent findings indicate that the β-adrenoceptor antagonist propranolol infused into the BLA also prevented the memory-enhancing effect of WIN55,212-2 (Hauer *et al*, 2010), it is thus possible that, comparable to the effects of glucocorticoids, cannabinoid effects on memory are also dependent on the availability of sufficient levels of arousal-induced noradrenergic activity within the BLA. On top of this direct interaction of WIN55,212-2 with arousal-induced noradrenergic mechanisms within the brain, the opposite effects of WIN55,212-2 on circulating corticosterone levels might add to the environmentally sensitive effects of cannabinoids on both short- and long-term recognition memory.

In summary, we provide evidence that cannabinoid effects on short- and long-term retention of recognition memory depend on the level of novelty-induced emotional arousal. These cannabinoid effects likely involve, at least in part, peripheral actions on modulating HPA-axis activity. These findings shed light on the contrasting effects of cannabinoid drugs on memory processing, thus providing new evidence that cannabinoid compounds can be either beneficial or detrimental to memory processes depending on the affective state of the individual at the time of drug consumption.

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CHAPTER 4

THE ENDOCANNABINOID SYSTEM MODULATES SPATIAL MEMORY RETRIEVAL DEPENDING ON THE LEVEL OF EMOTIONAL AROUSAL

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In preparation

Abstract

Compelling evidence indicates that cannabinoid drugs often induce biphasic effects on cognitive and emotional behavior depending on the aversiveness of the environmental context and the level of emotional arousal. It has been shown that variation in environmental aversiveness differentially influences spatial memory retrieval in rats and that glucocorticoids interact with the endocannabinoid system in impairing contextual aversive memory retrieval. Based on these findings, we investigated the role of the hippocampal endocannabinoid system on spatial memory retrieval in rats under two experimental conditions that differed with respect to their training- and test-associated arousal levels. To this aim male adult Sprague Dawley rats were trained in a Morris Water Maze task at two different water temperatures (19° C and 25° C) in order to elicit different levels of emotional arousal. First, we examined the arousal effects on spatial memory in vehicle treated rats and found that they showed a better retention and retrieval of the behavioral task. In a second set of experiments, 60 min before the retrieval trial the cannabinoid agonist WIN55,212-2 was bilaterally infused into the hippocampus. We found that WIN55,212-2 impaired memory retrieval only in rats trained under the high arousing condition (19° C) and such effect was blocked by a concurrent infusion with a nonimpairing dose of the cannabinoid type 1 receptor (CB1) antagonist AM251. In a third set of experiments, by employing the same experimental protocol, we examined whether the endogenous cannabinoid anandamide (AEA) and/or 2-arachidonoylglycerol (2-AG) could modulate spatial memory retrieval as well. To this aim the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor JZL184 were infused bilaterally into the hippocampus 60 min before the retrieval trial. We found that URB597 did not alter spatial memory retrieval performances in any of the two experimental conditions. Interestingly, highly comparable with WIN55,212-2 effects, JZL184 impaired spatial memory retrieval only in rats trained at the lower, high-arousing, temperature *via* an interaction with CB1 receptors. Consistently, in the high aroused rats, we found an increase in hippocampal 2-AG levels, but not AEA, after the training and probe trials, and alterations in CB1 affinity and in the activity of the main 2-AG degradative enzyme after the probe. The present findings indicate that the hippocampal endocannabinoid system plays a key role in mediating emotional arousal effects on spatial memory retrieval, shedding light on the neurobiological mechanism involved in the differential impact of stress on memory processes**.**

Introduction

Growing evidence demonstrates that the endocannabinoid system is crucially involved in the modulation of cognitive functions (Akirav, 2011; Campolongo *et al*, 2009b; Kano *et al*, 2009; Marsicano and Lafenetre, 2009; Wotjak, 2005). The main constituents of the endocannabinoid system are the cannabinoid receptor type 1 and type 2 (CB1 and CB2, respectively) (Devane *et al*, 1992; Herkenham *et al*, 1990; Matsuda *et al*, 1990) and the two major endogenous ligands for these receptors, the *N*-arachidonoyl ethanolamine (anandamide, AEA) (Devane *et al*, 1992) and the 2-arachidonoyl glycerol (2-AG) (Sugiura *et al*, 1995). In contrast to other classical neurotransmitters, AEA and 2-AG are not stored in vesicles at the presynapse, but are synthesised on demand and use a retrograde signaling by traveling from post- to presynaptic neurons where the cannabinoid receptors are expressed (Kano *et al*, 2009). Endocannabinoids bind to G protein-coupled CB1 receptors at presynaptic sites to regulate ion channel activity and neurotransmitter release (Kano *et al*, 2009). AEA and 2-AG are subsequently taken back into the cell by a still poorly defined uptake process mediated by a transporter mechanism (Fu *et al*, 2011; Hillard *et al*, 1997) and enzymatically degraded by the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Kano *et al*, 2009). CB1 receptors are highly expressed within cortico-limbic brain areas such as the basolateral complex of the amygdala (BLA), hippocampus and the prefrontal cortex (PFC) (Herkenham *et al*, 1991; Tsou *et al*, 1998) where they crucially modulate learning and memory processes for emotionally arousing experiences (Akirav, 2011; Campolongo *et al*, 2009b; Kano *et al*, 2009; Marsicano *et al*, 2009; Wotjak, 2005). Although there is a large number of contradictory reports in the literature regarding cannabinoid effects with respect to different memory phases (Morena and Campolongo, 2013) and the literature related to cannabinoid effects on retrieval is fairly limited, it seems that no controversy exists in this regard. Detrimental effects of cannabinoid receptor agonism on memory retrieval have been reported when administered either systemically (Mishima *et al*, 2001; Niyuhire *et al*, 2007) or in discrete brain areas such as the hippocampus (Atsak *et al*, 2012; Piri and Zarrindast, 2011; Segev and Akirav, 2011) and the BLA (Segev *et al*, 2011), at least under the experimental conditions investigated thus far. We recently reported that cannabinoid effects on memory retrieval are strictly related to the aversiveness of the environmental conditions and on the level of stress at the

time of drug injection, in such a way that systemic injections of the CB1 receptor agonist WIN55,212-2 induces opposing results on short-term object recognition memory retrieval depending on the level of context-induced emotional arousal (Campolongo *et al*, 2013). It has been previously reported that the endocannabinoid system within the BLA (Campolongo *et al*, 2009b) and hippocampus (de Oliveira Alvares *et al*, 2010) is crucially involved in mediating glucocorticoid impairing effects on memory consolidation. Furthermore, we recently showed that glucocorticoids interact with endocannabinoids in impairing contextual aversive memory retrieval (Atsak *et al*, 2012). It is well established that glucocorticoids have multiple effects on the different memory phases (Lupien and McEwen, 1997). The effects of stress and glucocorticoids on memory follow an inverted U-shaped dose-response relationship: extreme low and high levels may impair consolidation, but intermediate doses enhance memory (Mendl, 1999; Sandi and Pinelo-Nava, 2007; Yerkes, 1908). Likewise, it has been shown that variation in environmental aversiveness differentially influences spatial memory processes in rats (Akirav *et al*, 2004; Salehi *et al*, 2010). Salehi and coworkers (2010) induced different levels of stress in rats trained in a radial arm water maze task using three water temperatures (25°C, 19°C, and 16°C), which elicited increased plasma corticosterone levels: An inverted-U shape effect was found, with animals trained at 19°C showing a better memory retention than animals trained at either higher (16°C) or lower (25°C) stress conditions (Salehi *et al*, 2010). Once memories are consolidated, the efficacy or accuracy of the information retrieved remains vulnerable to glucocorticoids at the time of recall. Indeed stress or exogenous glucocorticoid administration typically impair memory of contextual/spatial information in rats and declarative information in human subject (de Quervain *et al*, 2009; de Quervain *et al*, 1998; de Quervain *et al*, 2000; Roozendaal *et al*, 2004a; Roozendaal *et al*, 2003; Roozendaal *et al*, 2004b). We recently demonstrated that glucocorticoids impair contextual fear memory retrieval by interacting with the endocannabinoid 2-AG within the hippocampus (Atsak *et al*, 2012), a brain region importantly implicated in the processing of spatial and contextual information (Meck *et al*, 1984; Morris *et al*, 1982; Moser *et al*, 1993). Based on the evidence summarized above, we investigated the role of the hippocampal endocannabinoid system on spatial memory retrieval in rats under two experimental conditions that differed with respect to their environment-induced stress level. Rats were divided into two main groups (high aroused and

low aroused) and trained in a Morris water maze task under two different water temperatures (19°C and 25°C) in order to elicit different levels of stress. In the first set of experiments we pharmacologically modulated the hippocampal endocannabinoid system to evaluate its putative role in the modulation of spatial memory retrieval under the two different stressful conditions. We next investigated whether there could be any alteration of endocannabinoid release in response to different stressful conditions with the final aim to evaluate how such alteration might underlie differential cannabinoid drug effects on spatial memory retrieval. Therefore, in the second part of this study hippocampal AEA and 2-AG levels were measured after training and probe trials in high aroused, low aroused and naïve (home-cage) rats. We further analyzed whether such changes could be related to any alteration of the cannabinoid hydrolytic enzymatic machinery or to the hippocampal CB1 receptor functionality.

Materials and Methods

Animals. Male adult Sprague-Dawley rats (320-370 g at the time of behavioral experiments; Charles River Laboratories, Calco, Italy) were individually housed in a temperature-controlled (20±1°C) vivarium and maintained under a 12 h light/dark cycle (07:00 AM-07:00 PM lights on). Food and water were available *ad libitum*. Training and testing were performed during the light phase of the cycle between 10:00 AM to 04:00 PM. All experimental procedures were in compliance with the guidelines of the U.S. National Institutes of Health and the Italian Ministry of Health (D.L. 116/92) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Surgery. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and were subsequently injected with 3 mL of saline (s.c.) to facilitate clearance of these drugs and prevent dehydration. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), and 2 stainless-steel guide cannulae (23 gauge) were implanted bilaterally, with the cannula tips 1.5 mm above the CA1 region of the dorsal hippocampus [11 mm; coordinates: AP, -3.4 mm; ML, ±1.8 mm; DV, -2.7 mm] according to the atlas of Paxinos and Watson (Paxinos and Watson, 2005). The cannulae were affixed to the skull with 2 anchoring screws and dental cement. Stylets (11-mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency. After surgery, the rats were retained in an

incubator until recovered from anesthesia and were then returned to their home cages. Rats were allowed to recover from surgery for 10 days before training. The rats were handled 1 min per day for 3 days before training.

Water maze task and experimental procedures. The experimental apparatus was a circular, black galvanized tank, 1.83 m in diameter and 0.6 m in height, filled with water to a depth of 20 cm. The maze was located in a room containing many salient, visual, extra-maze cues. A rectangular hidden platform (20 cm x 25 cm) was placed at a fixed location 25 cm away from the edge of the pool and 2.5 cm below the water. We used a slightly modified procedure of one previously described (Campolongo *et al*, 2009a). On each day of training, the rats were carried from the vivarium to the laboratory, and training began 120 min later. For spatial training, the rats were given four trials on each daily session for 2 consecutive days. This relatively small number of trials was chosen such that retention performance of control animals was moderate and drug administration could either enhance or impair performance. Before the first training trial, the rat was placed directly on the submerged platform for 15 sec. On each of the trials the rat was placed into the tank at one of the four designated starting points and allowed to find and escape onto the platform. If an animal failed to find the platform within 60 sec, it was manually guided to the platform. After mounting the platform, the rat was allowed to remain there for 10 sec and then it was placed into a holding cage for 25 sec until the start of the next trial. The time each rat spent to reach the platform was recorded as the escape latency. Retention of the spatial training was assessed 24 hr after the last training session with a 60 sec free-swim probe trial using a new starting position. The probe trial was videotaped and an automated tracking system (Panlab S.L.U., Varese, Italy) analyzed the swim path of each subject and calculated several corresponding dependent measures such as time spent in the quadrant containing the platform during training (target quadrant), time spent in the quadrant opposite to the target quadrant (opposite quadrant), initial latency to cross the platform location, number of crossings through the platform location, and total swim distance. The target and opposite quadrants were equidistant from the starting position used on the probe trial. In order to induce different experimental conditions with respect to their context-associated stress intensity, separate groups of animals were trained and tested at different water temperatures (25°C or 19°C) which

were previously shown to be appropriate temperatures to elicit different stress levels in rats (Akirav *et al*, 2004).

Drug treatment. The CB1 agonist WIN55,212-2 (10 or 30 ng in 0.5 µl), the FAAH inhibitor URB597 (10 or 30 ng in 0.5 µl) or the MAGL inhibitor JZL184 (0.1 or 1 µg in 0.5 µl) were administered into the hippocampus. To examine whether the effects of WIN55,212-2 and JZL184 were mediated *via* a selective activation of CB1 receptors, in other groups of rats the effective dose of WIN55,212-2 (10 ng in 0.5 µl) or JZL184 (1 µg in 0.5 µl) was infused either alone or concurrently with a non-impairing dose of the CB1 receptor antagonist AM251 (0.28 ng in 0.5 µl). Each dose was chosen on the basis previous pilot experiments conducted in our laboratory. All drugs were dissolved in a vehicle containing 5% Polyethylene glycol, 5% Tween80 and 90% saline and administered in the hippocampus 60 min before the probe trial in order to test animals for spatial memory retrieval. Bilateral infusions of drugs or an equivalent volume of vehicle into this brain region were given 60 min before the probe trial by using a 30 gauge injection needle connected by polyethylene tubing (PE-20) to a 10 µl Hamilton microsyringe driven by a minipump (KD Instruments, Varese, Italy). The injection needle protruded 1.5 mm beyond the tip of the cannula and a 0.5 µl injection volume/hemisphere was infused at a rate of 0.37 µl/min. The injection needles were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volume for the hippocampus was based on findings that infusions of this volume administered into the dorsal hippocampus produce different effects on the two-phase inhibitory avoidance task (Malin and McGaugh, 2006). All drugs were purchased from Tocris Bioscience and freshly prepared before each experiment.

Histology. The rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with a 0.9% saline solution. The brains were then removed and immersed in a 4% formaldehyde solution. At least 48-h before sectioning, the brains were transferred to a 20% sucrose solution in saline for cryoprotection. Coronal sections of 35 µm were cut on a cryostat, mounted on gelatin coated slides, and stained with cresyl violet. The sections were examined under a light microscope (Microscope Nikon 801, Florence, Italy), and the location of infusion needle tips in the hippocampus was made according to the standardized atlas plates of Paxinos and Watson (Paxinos *et al*, 2005) by an

observer blind to drug treatment condition. For all experiments, only rats with needle tips within the boundaries of the hippocampus were included in the data analysis. Approximately 14% of the animals were excluded from analysis because of either cannula misplacement or damage to the targeted tissue.

Endocannabinoid extraction and analysis. AEA and 2-AG endocannabinoid levels in the hippocampus were measured 20 min after the two days of training and immediately after the probe on naïve rats trained (and tested) under different water temperatures (25°C or 19°C). After rapid decapitation, the hippocampus was dissected within 3 min and stored at -80°C. Brain samples were subjected to a lipid extraction process and the endocannabinoid content of the lipid extracts was determined using isotope-dilution, liquid chromatography–mass spectrometry as previously described (Hauer *et al*, 2011).

Membrane preparation. Immediately after the probe trial, following rapid decapitation, the hippocampus was dissected from rats trained and tested under the high arousing or the low arousing conditions and from home-cage rats. Brain samples were stored at -80°C. Membranes were collected from hippocampi by the homogenization of frozen tissue in 10 volumes of TME buffer (50 mM Tris HCl, pH 5 7.4; 1 mM ethylenediaminetetraacetic acid [EDTA], and 3 mM MgCl2). Homogenates were then centrifuged at 18,000g for 20 min and the resulting crude membrane fraction-containing pellet was resuspended in 10 volumes of TME buffer. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA).

CB1 receptor radioligand-binding assay. CB1 receptor agonist binding parameters were determined using radioligand binding as previously described (Lee and Hill). Bmax (maximal binding site density) and Kd (binding affinity) values were determined by nonlinear curve fitting of specific binding data to the single site binding equation using GraphPad Prism (GraphPad, LaJolla, CA, USA).

FAAH activity assay. FAAH activity from hippocampal membranes was measured by conversion of AEA labeled with [3H] in the ethanolamine portion of the molecule to [3H] ethanolamine preparations as reported earlier (Lee *et al*). The binding affinity of AEA for FAAH (Km) and maximal hydrolytic activity of FAAH (Vmax) values for this conversion were determined by fitting the data to the Michaelis–Menten equation using GraphPad Prism (GraphPad, LaJolla, CA, USA).

MAGL activity assay. MAGL activity was measured by conversion of 2-oleoylglycerol labeled with [3H] ([3H] 2-OG) in the glycerol portion of the molecule to [3H] glycerol preparations. Membranes were incubated in a final volume of 0.5 ml TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, 1.0 mM EDTA, and 300 nM URB597, pH 7.4) that contained 1.0 mg/ml fatty acid-free bovine serum albumin and 100,000 dpm [3H] 2-OG. Isotherms were constructed using six concentrations of 2-OG at concentrations between 10 µM and 500 µM. Incubation was carried out at 30°C and the enzymatic reaction was stopped by the addition of 2 ml of chloroform/methanol (1:2). After remaining in room temperature for 30 min with frequent mixing, 0.67 ml of chloroform and 0.6 ml of water were added and aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [3H] in 0.5 ml each of the aqueous and organic phases was determined by liquid scintillation counting and conversion of [3H] 2-OG to [3H] glycerol was calculated. The Km and Vmax values for this conversion were determined by fitting the data to a single-site Michaelis–Menten equation using GraphPad Prism (GraphPad, LaJolla, CA, USA).

Statistics. Water maze training data were analyzed with one or two-way ANOVAs with the 2 acquisition trainings as repeated measure. Water maze measurements on probe trial for vehicle treated rats were analyzed with unpaired t tests and two-way ANOVA. Water maze measurements on probe trial for all the other experimental groups were analyzed with one-, two-, or three-way ANOVAs. Endocannabinoid brain levels, CB1 binding, FAAH and MAGL activity parameters were analyzed with one-way ANOVAs. The source of the detected significances was determined by Tukey–Kramer *post hoc* tests. Data were expressed as mean ± SEM. *P* values of less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

Results

High arousal condition enhances spatial memory retention and retrieval in vehicle-treated rats

We first analyzed possible behavioral differences in rats trained and tested under the two different water temperatures (25 °C and 19 °C) during the training days (before any drug treatment), and during the probe trial (after vehicle infusions).

A one-way ANOVA as repeated measures on the mean escape latencies to find the hidden platform during the two training days revealed a significant effect of training days ($F_{(1,74)}$ = 203.03, P < 0.0001), confirming that the two groups of animal progressively learned the spatial location of the platform across the training sessions. Interestingly, ANOVA also revealed a significant training days x water temperature effect ($F_{(1,74)}$ = 7.38, P = 0.0082). *Post-hoc* analysis indicated that rats trained under the high arousal conditions (19 °C-water temperature) presented lower mean escape latencies on the second day of training as compared with rats trained at 25 °C (P < 0.01; Fig. 1), thus indicating that high

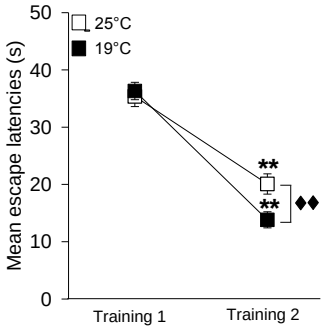

Figure 1. Effect of water temperature on training performances of vehicle treated rats. Both rats trained at 25 °C and 19 °C acquired the behavioral task as indicated by a significant difference between mean escape latencies during training 1 and training 2. Rats trained at 19 °C showed a better memory performance that rats at 25 °C during training 2. ** P < 0.01 vs training 1; ◆◆, P < 0.01 between mean escape latencies at 25 °C vs mean escape latencies at 19 °C. Results represent mean ± SEM (n = 36-40 per group).

aroused rats had a better memory retention of the task. As shown in Fig. 2 the high arousal condition improved spatial memory retrieval in rats. Unpaired *t*-tests revealed that rats under the high arousal condition spent less time to initially cross the platform location (t_{74}= -3.72; P = 0.0004; Fig. 2A) and performed a higher number of crossings through the platform location (t_{74}= 2.97; P = 0.0041; Fig. 2B) as compared to low aroused rats. The pattern of effects for quadrant search times was similar to that for initial latency to cross the platform location and number of target crossings (Fig. 2C). A two-way ANOVA for time spent in searching the platform location revealed no water temperature effect ($F_{(1,148)}$ = 0.85, P = 0.36) but a significant quadrant ($F_{(1,148)}$ = 171.18, P < 0.0001) and water temperature x quadrant interaction effect ($F_{(1,148)}$ = 4.30, P = 0.039). Both groups of vehicle-infused rats acquired correctly the behavioral task. Indeed they exhibited memory of the platform position during trainings, as indicated by significantly longer search times in the vicinity of the platform location (i.e., in the target quadrant) than in the opposite quadrant (P < 0.01; Fig. 2C). Interestingly, rats under the lower, high arousing, water temperature spent significantly less time in the

opposite quadrant than rats under the higher water temperature ($P < 0.01$; Fig. 2C).

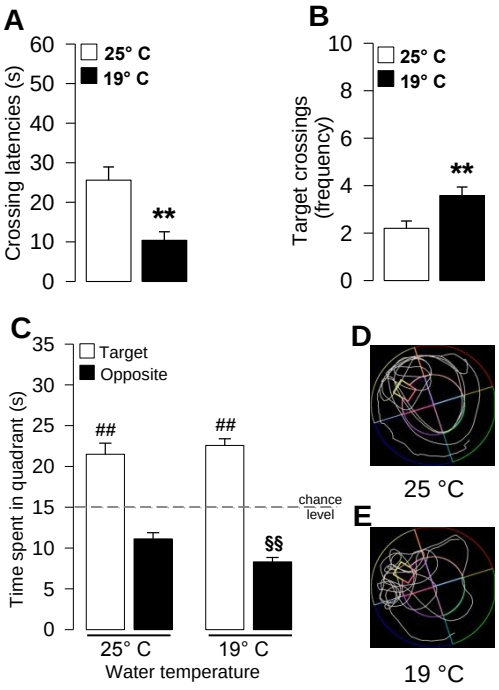

Figure 2. The high arousal condition (19 °C) enhanced spatial memory retrieval in rats. High aroused rats (19° C) spent less time to first cross the platform location (A) and performed a higher number of target crossings (B) during probe as compared with the low aroused group (25 °C). (C) Both experimental groups spent more time in the target quadrant than in the opposite quadrant in searching the platform location. High aroused rats spent less time in the opposite quadrant that low aroused rats. Representative probe trial swim paths of low aroused (D) and high aroused rats (E). **, $P < 0.01$ vs low aroused group; ##, $P < 0.01$ vs the correspondent opposite quadrant; §, $P < 0.05$ vs opposite quadrant time of low aroused group. All results represent mean ± SEM (n = 36-40 per group).

To examine possible differences in motor performance that may have been caused by the different water temperatures, the swimming distances during the probe trial of the different groups were compared. No significant differences were found between swimming distances of the two experimental groups ($t_{74} = 0.35$; $P = 0.73$; data not shown).

Intra-hippocampal infusion of the CB receptor agonist WIN55,212–2 impairs spatial memory retrieval through the activation of the CB1 receptor under high arousal but not low arousal condition

This experiment investigated whether intra-hippocampal infusions of the cannabinoid receptor agonist WIN55,212-2 60 min before the probe trial altered spatial memory retrieval and whether this WIN55,212-2 effect was influenced by the different water temperatures.

25° C-water temperature. All rats learned to locate the platform position during the days of training before drug treatment, as indicated by decreasing mean

escape latencies as training progressed ($F_{(1,32)}$ = 95.96, P < 0.0001); data not shown). As shown in Fig. 3A-B-C, intra-hippocampal infusions of WIN55,212-2 60 min before the probe trial did not induce any alteration on spatial memory retrieval in rats under the low arousal conditions. One-way ANOVAs for the initial latency to cross the platform location (i.e. crossing latencies) and for the number of crossings through the platform location (target crossings) did not reveal a significant WIN55,212-2 effect ($F_{(2,32)}$ = 0.50, P = 0.62; $F_{(2,32)}$ = 0.52, P = 0.60; respectively, Fig. 3A-B). A two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,64)}$ = 59.30, P < 0.0001), but did not reveal a significant drug effect ($F_{(2,64)}$ = 0.13, P = 0.88), or an interaction between both factors ($F_{(2,64)}$ = 0.65, P = 0.52). *Post-hoc* analysis revealed that all the experimental groups spent longer search times in the target quadrant than in the opposite quadrant (P < 0.01; Fig 3C), thus indicating that animals exhibited memory of the platform position during training. To examine possible differences in motor performance that may have been caused by the different drug treatments, the total swim distances during the probe trial of the different groups were compared. No significant differences were found between total swim distances of the experimental groups ($F_{(2,32)}$ = 0.77; P = 0.47; data not shown).

19° C-water temperature. All animals progressively learned the spatial location of the platform across the training sessions before drug treatment, as indicated by decreasing mean escape latencies as training progressed ($F_{(1,31)}$ = 140.88, P < 0.0001; data not shown). Figures 3D-E-F show that intra-hippocampal infusions of WIN55,212-2 60 min before the probe trial impaired spatial memory retrieval in rats under the high arousal condition. One-way ANOVAs for crossing latencies and for target crossings during the probe trial revealed a significant WIN55,212-2 effect ($F_{(2,31)}$ = 3.53, P = 0.041; $F_{(2,31)}$ = 5.78, P = 0.0074; respectively, Fig. 3D-E). The 10 ng/0.5 µl dose of WIN55,212-2, but not the higher dose, significantly increased the crossing latencies (P < 0.05; Fig. 3D) and, conversely, decreased the number of crossings through the platform location (P < 0.01; Fig. 3E) of rats trained and tested at the lower temperature as compared with the respective control groups. A two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,62)}$ = 109.23, P < 0.0001), no drug effect ($F_{(2,62)}$ = 9.23, P = 0.56), and a significant interaction between both factors ($F_{(2,62)}$ = 7.37, P = 0.0013). *Post-hoc* analysis revealed that all the experimental groups spent longer time swimming in the target quadrant than in the opposite quadrant (P < 0.01; Fig. 3F), thus indicating that animals exhibited memory of the platform position

during trainings. Further, time spent by rats administered with WIN55,212-2 (10-30 ng/0.5 µL) in searching the platform location in the target quadrant was shorter than that of vehicle-treated group ($P < 0.05$; Fig. 3F). No significant differences were found between total swim distances of the three experimental groups during the probe trial ($F_{(2,31)} = 0.46$; $P = 0.63$; data not shown), thus demonstrating that drug infusions did not alter locomotor activity in rats under the high arousal condition.

To investigate whether hippocampal CB1 receptors might mediate the impairing effect of WIN55,212-2 treatment on retrieval of spatial memory, bilateral infusions of a non-impairing dose of the CB1 receptor antagonist AM251 (0.28 ng in 0.5 µl) were concurrently administered into the dorsal hippocampus 60 min before retention testing together with the effective dose of WIN55,212-2 (10 ng/0.5 µl) or with vehicle. Repeated-measures ANOVA for mean escape latencies during trainings showed that all groups acquired the Morris water maze task as indicated by decreasing latencies to find the hidden platform between days ($F_{(1,44)} = 209.55$; $P < 0.0001$; data not shown). As shown in Fig. 3G-H, two-way ANOVAs for crossing latencies and number of target crossings during testing for memory retrieval revealed a significant main effect of WIN55,212-2 ($F_{(1,44)} = 8.99$; $P = 0.0044$; $F_{(1,44)} = 5.37$; $P = 0.025$; for crossing latencies and target crossings, respectively), no AM251 effect ($F_{(1,44)} = 3.43$; $P = 0.071$; $F_{(1,44)} = 2.81$; $P = 0.10$; for crossing latencies and target crossings, respectively) and a significant interaction effect between these two treatments ($F_{(1,44)} = 5.59$; $P = 0.023$; $F_{(1,44)} = 7.22$; $P = 0.010$; for crossing latencies and target crossings, respectively). Tukey's *post hoc* comparisons showed that intra-hippocampal infusions of WIN55,212-2 alone significantly increased the initial time to cross the platform location ($P < 0.01$; Fig. 3G) and decreased the number of target crossings ($P < 0.05$; Fig. 3H) as compared with rats given vehicle. Initial crossing latencies of rats given WIN55,212-2 together with AM251 were significantly shorter than those of rats given WIN55,212-2 alone ($P < 0.05$; Fig. 3G) and equivalent to those of rats given vehicle and AM251 alone. Consistently, the number of target crossings of rats given WIN55,212-2 with AM251 were significantly higher than those of rats given WIN55,212-2 alone ($P < 0.05$; Fig. 3H) and equivalent to those of rats given vehicle and AM251 alone.

A three-way ANOVA for quadrant search times during the probe trial revealed no main effect of WIN55,212-2 ($F_{(1,88)} = 1.18$; $P = 0.28$) but a significant AM251 ($F_{(1,88)} = 4.95$; $P = 0.029$), quadrant ($F_{(1,88)} = 191.75$; $P < 0.0001$) and WIN55,212-2 x AM251

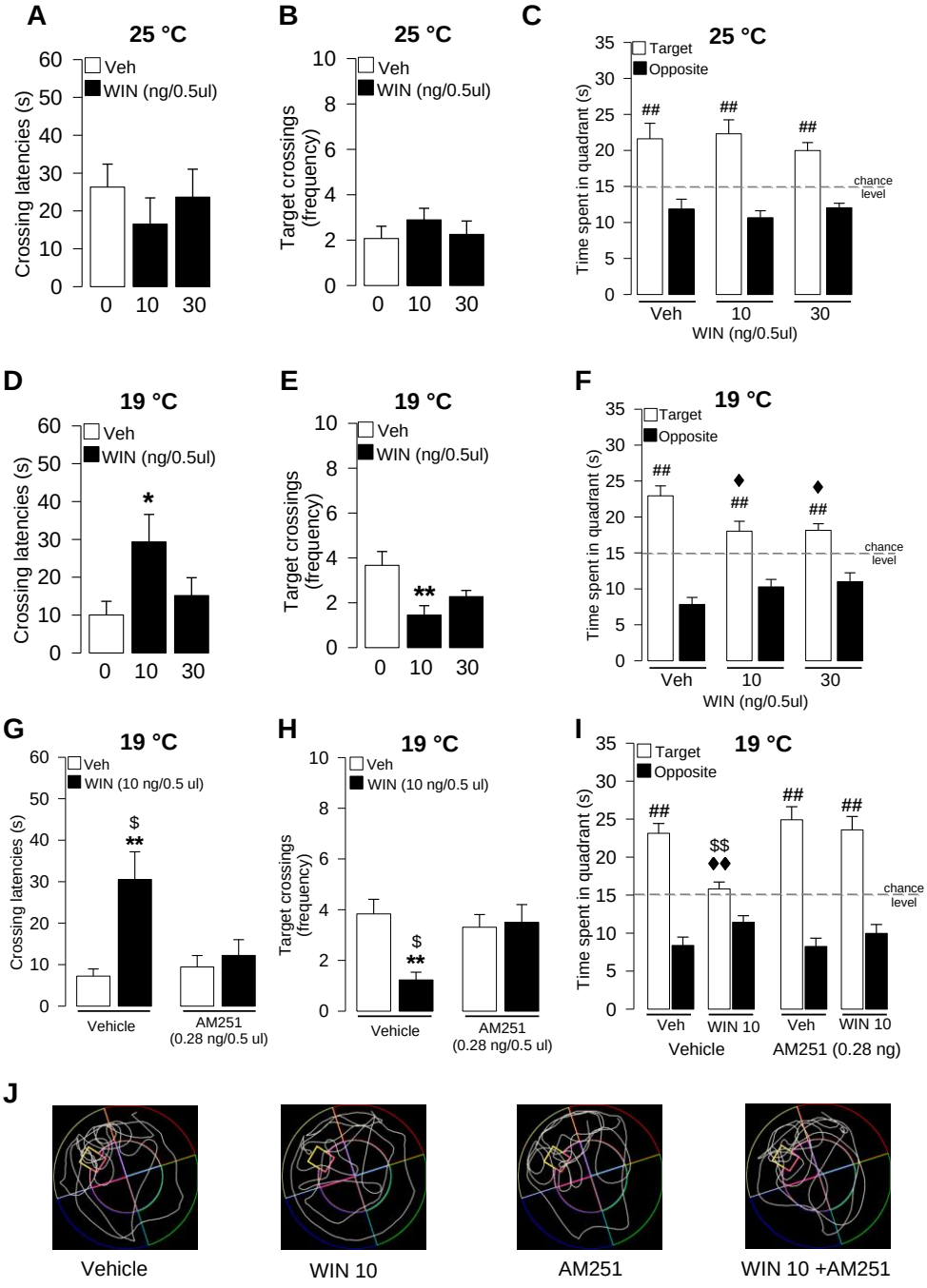

Figure 3. Intra-hippocampal infusions of the cannabinoid agonist WIN55,212-2 (WIN) did not affect spatial memory retrieval in the low arousal condition (25 °C), but did impair it in the high arousal condition (19 °C). The CB1 antagonist AM251 (0.28 ng/0.5 µl) blocked the memory impairing effect

induced by a concurrent administration with the effective dose of WIN (10 ng/0.5 µl; WIN 10) under the high arousal condition. Effects of WIN on the latency to first cross the platform location (A), the number of target crossings (B) and the time spent in the target and opposite quadrants (C) during the 1-min probe trial at 25 °C. Effects of WIN on the latency to first cross the platform location (D), the number of target crossings (E) and the time spent in the target and opposite quadrants (F) during the 1-min probe trial at 19 °C. Effects of WIN+AM251 administration on the latency to first cross the platform location (G), the number of target crossings (H) and the time spent in the target and opposite quadrants (I) during the 1-min probe trial at 19 °C. (J) Representative probe trial swim paths. *, $P < 0.05$, **, $P < 0.01$ vs the correspondent vehicle (veh) group; ##, $P < 0.01$ vs the correspondent opposite quadrant; ◆, $P < 0.05$, ◆◆, $P < 0.01$ vs target quadrant time of vehicle group; $, $P < 0.05$, $$, $P < 0.01$ vs the correspondent WIN+AM251 group. All results represent mean ± SEM (n = 9-14 per group).

x quadrant interaction effect ($F_{(1,88)} = 4.20$; $P = 0.043$; Fig. 3I). Control rats given vehicle infusions into the hippocampus spent significantly more time in the target quadrant than in the opposite quadrant ($P < 0.01$; Fig. 3I). As was found in the previous experiment, WIN55,212-2 infused into the hippocampus 60 min before probe decreased time spent in the target quadrant ($P < 0.01$, compared with vehicle; Fig. 3I). Although pretest administration of AM251 into the hippocampus alone did not affect time spent in either the target or the opposite quadrants, AM251 blocked the changes induced by co-infusion with WIN55,212-2 into the hippocampus. Rats given combined infusions of AM251 and WIN55,212-2 spent significantly more time in the target quadrant ($P < 0.01$; Fig. 3I) than rats given WIN55,212-2 alone and on this measure did not differ significantly from control rats given either vehicle or AM251 infusions into the hippocampus. Two-way ANOVA for total swim distance did not reveal significant WIN55,212-2 ($F_{(1,44)} = 0.006$; $P = 0.94$), AM251 ($F_{(1,44)} = 0.34$; $P = 0.56$), or WIN55,212-2 x AM251 interaction effects ($F_{(1,44)} = 0.13$; $P = 0.72$; data not shown).

Intra-hippocampal infusion of the AEA hydrolysis inhibitor URB597 does not affect spatial memory retrieval of rats neither in the low arousal nor in the high arousal conditions

We next examined whether the endogenous cannabinoid AEA could modulate spatial memory retrieval. To this aim the FAAH inhibitor URB597 was bilaterally infused into the hippocampus 60 min before the probe trial in rats trained and tested under different water temperatures.

25° C-water temperature. All rats learned to locate the platform position during the trainings before drug treatment, as indicated by decreasing mean escape latencies as training progressed ($F_{(1,33)} = 86.99$, $P < 0.0001$); data not

shown). As shown in Fig. 4A-B-C, intra-hippocampal infusions of URB597 60 min before the probe trial did not induce any alteration on spatial memory retrieval in rats under the low arousal condition. One-way ANOVAs for the initial latency to cross the platform location and for the number of crossings through the platform location did not reveal a significant URB597 effect ($F_{(2,33)}$ = 0.077, P = 0.93; $F_{(2,33)}$ = 0.69, P = 0.51; respectively, Fig. 4A-B). A two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,66)}$ = 112.69, P < 0.0001), but did not reveal a significant drug effect ($F_{(2,66)}$ = 0.16, P = 0.86), or an interaction between both factors ($F_{(2,66)}$ = 2,35, P = 0.10). *Post-hoc* analysis revealed that all the experimental groups spent longer time in the target quadrant than in the opposite quadrant (P < 0.01; Fig. 4C), thus indicating that they exhibited memory of the platform position during trainings. No significant differences were found between total swim distances of the experimental groups ($F_{(2,66)}$ = 0.053; P = 0.95; data not shown).

19° C-water temperature. A one-way ANOVA as repeated measures on the mean escape latencies to find the hidden platform on the two training days revealed a significant effect of training days ($F_{(1,28)}$ = 84.23, P < 0.0001 data not shown), confirming that rats progressively learned the spatial location of the platform across the training sessions. Figures 4D-E-F shows that intra-hippocampal infusions of URB597 60 min before the probe trial did not alter spatial memory retrieval in rats under the high arousal conditions. One-way ANOVAs for crossing latencies and for target crossings during the probe trial did not reveal significant drug effects ($F_{(2,28)}$ = 0.36, P = 0.70; $F_{(2,28)}$ = 0.62, P = 0.55; respectively, Fig. 4D-E). A two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,56)}$ = 82.64, P < 0.0001), but no drug ($F_{(2,56)}$ = 0.084, P = 0.92), or quadrant x drug effects ($F_{(2,56)}$ = 1.56, P = 0.22). *Post-hoc* analysis revealed that all the experimental groups spent longer time swimming in the target quadrant than in the opposite quadrant (P < 0.01; Fig. 4 F), thus indicating that animals exhibited memory of the platform position during training. No significant differences were found between total swim distances of the three experimental groups during the probe trial ($F_{(2,28)}$ = 0.52; P = 0.60; data not shown).

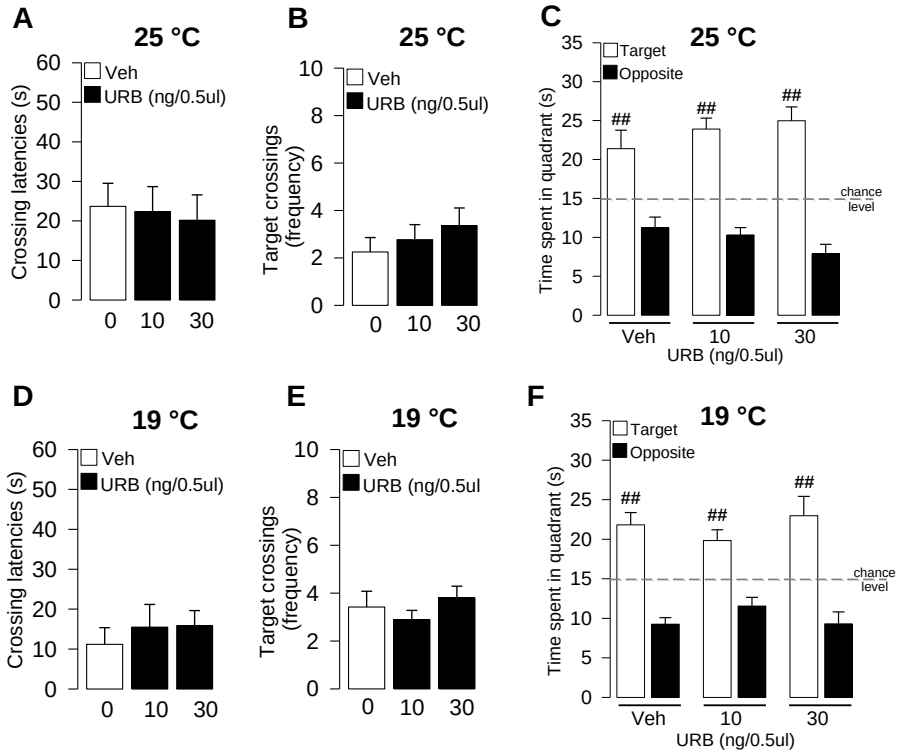

Figure 4. Intra-hippocampal infusions of the FAAH inhibitor URB597 (URB) did not affect spatial memory retrieval in either of the two different arousing conditions. Effects of URB on the latency to first cross the platform location (A), the number of target crossings (B) and the time spent in the target and opposite quadrants (C) during the 1-min probe trial at 25 °C. Effects of URB on the latency to first cross the platform location (D), the number of target crossings (E) and the time spent in the target and opposite quadrants (F) during the 1-min probe trial at 19 °C. ##, $P < 0.01$ vs the correspondent opposite quadrant. All results represent mean ± SEM (n = 9-13 per group).

Intra-hippocampal infusion of the 2-AG hydrolysis inhibitor JZL184 impairs spatial memory retrieval through the activation of the CB1 receptor under high arousal but not low arousal conditions

Given the previous results, we further examined whether the endogenous cannabinoid 2-AG could modulate spatial memory retrieval as well. To this aim the MAGL inhibitor JZL184 was bilaterally infused into the hippocampus 60 min before the probe trial in rats trained and tested under different water temperatures.

25° C-water temperature. All the experimental groups learned to find the platform position during the two days of training, before drug treatment, as indicated by

decreasing mean escape latencies between training days ($F_{(1,36)}$ = 114.30, P < 0.0001; data not shown). As shown in Fig. 5A-B-C, intra-hippocampal infusions of JZL184 60 min before the probe trial did not induce any alteration on spatial memory retrieval in rats under the low arousal conditions. Initial latencies to cross the platform location and the number of target crossings of rats in this experiment are presented in Fig. 5A-B. One-way ANOVAs did not reveal a significant JZL184 effect ($F_{(2,36)}$ = 0.24, P = 0.79; $F_{(2,36)}$ = 0.37, P = 0.69; for crossing latencies and target crossings, respectively, Fig. 5A-B). A two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,72)}$ = 47.31, P < 0.0001), but did not reveal a significant drug effect ($F_{(2,72)}$ = 0.45, P = 0.64), or an interaction between both factors ($F_{(2,72)}$ = 0.19, P =0.83). *Post-hoc* analysis revealed that all the experimental groups spent longer search times in the vicinity of the platform location than in the opposite quadrant (P < 0.01; Fig. 5 C), thus indicating that animals exhibited memory of the platform position during trainings. To examine possible differences in motor performance that may have been caused by the different drug treatments, the total swim distance during the probe trial of the different groups were compared. No significant differences were found between total swim distances of the three experimental groups ($F_{(2,36)}$ = 0.008; P = 0.99; data not shown).

19° C-water temperature. All animals progressively learned the spatial location of the platform across the training sessions before drug treatment, as indicated by decreasing mean escape latencies as training progressed ($F_{(1,26)}$ = 119.11, P < 0.0001; data not shown).

Highly comparable with WIN55,212-2 effects, Fig. 5D-E-F shows that intra-hippocampal infusions of JZL184 60 min before the probe trial impaired spatial memory retrieval in rats under the high arousal condition. One-way ANOVAs for crossing latencies and target crossings during the probe trial revealed a significant JZL184 effect ($F_{(2,26)}$ = 4.28, P = 0.025; $F_{(2,26)}$ = 5.99, P = 0.0073; for crossing latencies and target crossings, respectively, Fig. 5D-E). The 1 µg/0.5 µl dose of JZL184, but not the lower dose, significantly increased the crossing latencies (P < 0.05; Fig. 5D) and, conversely, both doses of JZL184 (0.1-1 µg/0.5 µl) decreased the number of crossings through the platform location (P < 0.05; Fig. 5E) of rats trained and tested at the lower temperature as compared with the respective control groups. As shown in Fig. 5F, a two-way ANOVA for quadrant search times during the probe trial showed a significant quadrant effect ($F_{(1,52)}$ = 59.30, P < 0.0001), no drug effect ($F_{(2,52)}$ = 0.17, P = 0.84), and a significant interaction

between both factors ($F_{(2,52)}$ = 7.05, *P* = 0.002). *Post hoc* analysis revealed that control rats given vehicle infusions into the hippocampus spent significantly more time in the target quadrant than in the opposite quadrant (*P* < 0.01; Fig. 5F), thus indicating that animals acquired the behavioral task. The 1 μg/0.5 μl dose of JZL184, but not the lower dose, infused into the hippocampus 60 min before retention testing decreased time spent in the target quadrant to a chance level and increased time spent in the opposite quadrant (*P* < 0.05 vs control group, for both comparisons; Fig. 5 F). No significant differences were found between total swim distances of the three experimental groups during the probe trial ($F_{(2,26)}$ = 2.44; *P* = 0.11; data not shown), thus demonstrating that drug infusion did not alter locomotor activity in rats under the high arousal condition.

This experiment examined whether CB1 receptor activation in the hippocampus may be essential in enabling JZL184 effects on memory retrieval. To this aim the CB1 receptor antagonist AM251 (0.28 ng/0.5 μl) was concurrently infused bilaterally into the dorsal hippocampus 60 min before retention testing together with the effective dose of JZL184 (1 μg/0.5 μl) or with vehicle.

All groups acquired the Morris water maze task as indicated by decreasing latencies to find the hidden platform between days ($F_{(1,41)}$ = 123.48; *P* < 0.0001; data not shown). Figures 5G-H-I shows the effect of intra-hippocampal infusions of AM251 (0.28 ng/0.5 μL) on probe trial retrieval impairment induced by intra-hippocampal infusion of JZL184. Two-way ANOVAs for crossing latencies and number of target crossings during testing for memory retrieval revealed significant JZL184 ($F_{(1,41)}$ = 9.56; *P* = 0.0036; $F_{(1,41)}$ = 5.90; *P* = 0.02; for crossing latencies and target crossings, respectively), AM251 ($F_{(1,41)}$ = 8.24; *P* = 0.0065; $F_{(1,41)}$ = 4.28; *P* = 0.045; for crossing latencies and target crossings, respectively) and JZL184 x AM251 interaction effects ($F_{(1,41)}$ = 9.79; *P* = 0.0032; $F_{(1,41)}$ = 10.55; *P* = 0.0023; for crossing latencies and target crossings, respectively). Intra-hippocampal infusion of JZL184 alone significantly increased the initial time to cross the platform location (*P* < 0.01; Fig. 5G) and decreased the number of target crossings (*P* < 0.01; Fig. 5H) as compared with rats given vehicle. Initial crossing latencies of rats given JZL184 together with AM251 were significantly shorter than those of rats given JZL184 alone (*P* < 0.01; Fig. 5G) and equivalent to those of rats given vehicle and AM251 alone. Consistently, the number of target crossings of rats given JZL184 with AM251 were significantly higher than those of rats given JZL184 alone (*P* < 0.01; Fig. 5H) and equivalent to those of rats given vehicle and AM251 alone. A three-way ANOVA for time spent in the target quadrant revealed

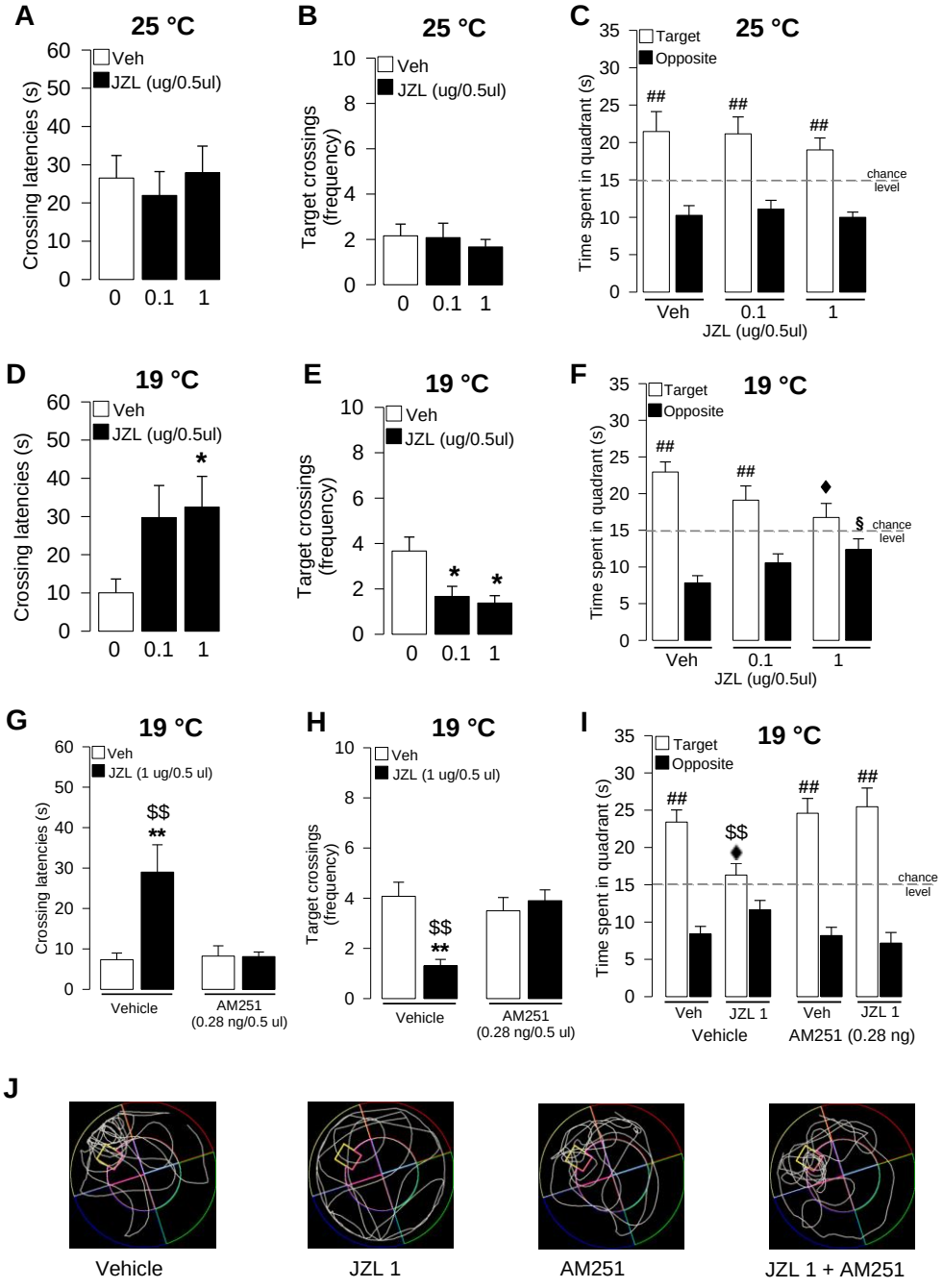

Figure 5. Intra-hippocampal infusions of the MAGL inhibitor JZL184 (JZL) did not affect spatial memory retrieval in the low arousal condition (25 °C), but did impair it in the high arousal condition (19 °C). The CB1 antagonist AM251 (0.28 ng/0.5 µl) blocked the memory impairing effect induced by

a concurrent administration with the effective dose of JZL (1 μg/0.5 μl; JZL 1) under the high arousal condition. Effects of JZL on the latency to first cross the platform location (A), the number of target crossings (B) and the time spent in the target and opposite quadrants (C) during the 1-min probe trial at 25 °C. Effects of JZL on the latency to first cross the platform location (D), the number of target crossings (E) and the time spent in the target and opposite quadrants (F) during the 1-min probe trial at 19 °C. Effects of JZL+AM251 administration on the latency to first cross the platform location (G), the number of target crossings (H) and the time spent in the target and opposite quadrants (I) during the 1-min probe trial at 19 °C. (J) Representative probe trial swim paths. *, $P < 0.05$, **, $P < 0.01$ vs the correspondent vehicle (veh) group; ##, $P < 0.01$ vs the correspondent opposite quadrant; ◆, $P < 0.05$, vs target quadrant time of vehicle group; §, $P < 0.05$ vs opposite quadrant time of vehicle group; $$, $P < 0.01$ vs the correspondent JZL+AM251 group. All results represent mean ± SEM (n = 8-14 per group).

no main effects of JZL184 ($F_{(1,82)} = 0.76$; $P = 0.39$) or AM251 ($F_{(1,82)} = 1.52$; $P = 0.22$) on quadrant search times during the probe trial (Fig. 5I). However, there was a significant quadrant ($F_{(1,82)} = 141.89$; $P < 0.0001$) and JZL184 x AM251 x quadrant interaction effect ($F_{(1,82)} = 7.18$; $P = 0.0089$). Control rats given vehicle infusions into the hippocampus spent significantly more time in the target quadrant than in the opposite quadrant ($P < 0.01$; Fig. 5I). As was found in the previous experiment, JZL184 infused into the hippocampus 60 min before probe decreased time spent in the target quadrant ($P < 0.05$, compared with vehicle; Fig 5I). Although pretest administration of AM251 into the hippocampus alone did not affect time spent in either the target or the opposite quadrant, AM251 blocked the changes induced by coadministration of JZL184 into the hippocampus. Rats concurrently infused with AM251 and JZL184 spent significantly more time in the target quadrant ($P < 0.01$; Fig. 5I) than rats given JZL184 alone without significantly differing from control rats given either vehicle or AM251 alone. Two-way ANOVA for total swim distance did not reveal significant JZL184 ($F_{(1,41)} = 0.008$; $P = 0.93$), AM251 ($F_{(1,41)} = 1.69$; $P = 0.20$), or JZL184 x AM251 interaction effects ($F_{(1,41)} = 1.21$; $P = 0.28$; data not shown).

High arousal but not low arousal condition increases hippocampal 2-AG levels during spatial memory consolidation and retrieval of the Morris water maze task

Previous findings have shown that rats, 20 min after a Morris water maze training at 19 °C water temperature, performed better and presented higher levels of plasma corticosterone than rats trained at 25 °C (Akirav *et al*, 2004). Therefore we investigated whether such increase in stress hormone levels could alter endocannabinoid hippocampal levels and, thus, modulate spatial memory processes. To this aim parallel groups of rats were trained at 19 °C or 25 °C water

temperature and sacrified 20 min after the two days of training for brain tissue dissection and subsequent endocannabinoid measurements in the hippocampus. To assess whether the possible alteration in endocannabinoid hippocampal levels might underlie the different effects in memory retrieval observed in the previous experiments, other groups of rats were sacrified immediately after the 1-min probe trial. Control rats (home cage) were only handled and sacrified at the same time points. We did not found any alteration in hippocampal AEA levels in any of the experimental groups. One-way ANOVAs for hippocampal AEA levels did not reveal any experimental condition effect ($F_{(2,22)}$ = 1.03; *P* = 0.37; $F_{(2,23)}$ = 1.46; *P* = 0.25; $F_{(2,25)}$ = 0.94; *P* = 0.40, for training 1, training 2 and probe, respectively; Table 1). However, as shown in Table 1, one-way ANOVAs for hippocampal 2-AG levels

Table 1. Hippocampal endocannabinoid concentration of low aroused (25 °C), high aroused (19 °C) and home cage rats.

Time point	Anandamide (pmol/g tissue)			2-Arachidonoylglycerol (nmol/g tissue)		
	Home cage	19 °C	25 °C	Home cage	19 °C	25 °C
20 min after training 1	5.8 ± 0.4	7.1 ± 0.5	7.3 ± 1.5	4.3 ± 0.4	6.7 ± 1.0*,#	3.6 ± 0.3
20 min after training 2	8.7 ± 1.7	6.7 ± 0.9	5.9 ± 0.8	3.5 ± 0.3	5.3 ± 0.4**,#	3.9 ± 0.3
Immediately after probe	7.8 ± 1.3	7.0 ± 0.9	6.1 ± 0.4	4.7 ± 0.7	7.8 ± 1.1*,#	4.7 ± 0.5

*, *P* < 0.05, **, *P* < 0.01 vs the corresponding home cage group, #, *P* < 0.05 vs the corresponding 25 °C group. Data are expressed as mean ± SEM (n = 7 – 10 per group).

after trainings 1, training 2 and probe, revealed a significant experimental condition effect ($F_{(2,19)}$ = 6.14; *P* = 0.0088; $F_{(2,23)}$ = 7.91; *P* = 0.0024; $F_{(2,24)}$ = 4.68; *P* = 0.019, respectively). Rats trained at 19 °C presented higher levels of 2-AG than both rats in the 25 °C group and home cage rats as assessed 20 min after training 1 (*P* < 0.05; for both comparisons; Table 1), training 2 (*P* < 0.05 vs low aroused group; *P* < 0.01 vs home cage group; Table 1) and immediately after probe (*P* < 0.05; for both comparisons; Table 1). Thus, our findings that high arousal conditions elevates 2-AG levels in the hippocampus, suggest that hippocampal endocannabinoid signaling is critically involved in mediating the stress effects on retrieval of contextual fear memory.

High arousal and low arousal conditions modulates hippocampal CB1 receptor binding during spatial memory retrieval

This experiment investigates whether the level of stress associated to the different water temperatures might induce any alteration on hippocampal CB1 receptor binding during memory retrieval. Three different groups of rats (high aroused, low aroused and home cage) were sacrified immediately after the 1-min probe trial for hippocampal tissue dissection. As shown in Table 2, one-way ANOVA for CB1 receptor Bmax revealed a significant experimental condition effect ($F_{(2,15)} = 8.24$; $P = 0.0039$). Interestingly, both rats in the high arousal and low arousal conditions exhibited lower levels of the CB1 receptor binding site densities relative to home cage animals ($P < 0.01$; for both comparisons; Table 2), thus suggesting that the degree of emotional arousal associated to the behavioral task *per se*, independently from the different water temperatures, might induce such alterations. No main experimental condition effect was found for CB1 receptor binding affinity ($F_{(2,15)} = 1.64$; $P = 0.23$; Table 2).

Table 2. Bmax and Kd values for CB1 receptor radioligand binding assay and Vmax and Km values for FAAH and MAGL activity assay in low aroused (25 °C), high aroused (19 °C) and home cage rats.

Parameter	CB1 binding assay		
	Home cage	19 °C	25 °C
Bmax (pmol/mg protein)	1.6 ± 0.04	1.3 ± 0.05**	1.3 ± 0.1**
Kd (nM)	1.6 ± 0.3	1.3 ± 0.1	1.2 ± 0.1
	FAAH activity assay		
	Home cage	19 ° C	25 °C
Vmax (pmol/mg protein/min)	1961.3 ± 178.6	1710.0 ± 115.6	1889.8 ± 118.2
Km (nM)	0.84 ± 0.1	0.86 ± 0.1	0.85 ± 0.1
	MAGL activity assay		
	Home cage	19 ° C	25 °C
Vmax (nmol/mg protein/min)	61.5 ± 4.5	71.3 ± 7.7	62.8 ± 2.3
Km (nM)	40.5 ± 5.5	61.8 ± 4.9*,#	36.1 ± 4.1

*, $P < 0.05$, **, $P < 0.01$ vs the corresponding home cage group, #, $P < 005$ vs the corresponding 25 °C group. Data are expressed as mean ± SEM (n = 3- 6 per group).

High arousal but not low arousal condition modulates hippocampal MAGL activity during spatial memory retrieval, without altering the activity of FAAH

These experiments investigate whether the level of stress associated to the different water temperatures might induce any alteration on hippocampal FAAH and MAGL activity during memory retrieval. Three different groups of rats (high aroused, low aroused and home cage) were sacrified immediately after the 1-min probe trial for hippocampal tissue dissection.

As shown in Table 2, one-way ANOVAs for Vmax and Km of FAAH did not reveal any significant experimental condition effect ($F_{(2,15)} = 0.85$; $P = 0.45$; $F_{(2,15)} = 0.28$; $P = 0.76$; for Vmax and Km, respectively). Interestingly, one-way ANOVAs for Km, but not for Vmax, of MAGL did exhibit a significant experimental condition effect ($F_{(2,8)} = 7.04$; $P = 0.017$; $F_{(2,8)} = 1.12$; $P = 0.37$; for Km and Vmax, respectively; Table 2). *Post hoc* comparisons, indicated that the behavioral task performed at 19 °C water temperature increased the MAGL Km value as compared to those of home cage and low aroused rats ($P < 0.05$, for both comparisons; Table 2).

Discussion

In the present study we demonstrate that a pharmacologically-induced increase of hippocampal 2-AG levels mediates the impairment of spatial memory retrieval for a high emotionally arousing, but not a low emotionally arousing version of a Morris water maze task. Furthermore our findings indicate that the high emotionally arousing version of this behavioral task induce molecular changes in the endocannabinoid system components that mediate a better memory consolidation and retrieval in naïve animals.

We first evaluated spatial memory performance in naïve rats and, accordingly with previous findings (Akirav *et al*, 2001; Salehi *et al*, 2010; Sandi *et al*, 1997), we found that rats trained and tested under moderate level of stress (19°C water temperature) presented a better memory consolidation and retrieval than rats under the low arousing condition (25°C water temperature).

Bilateral intra-hippocampal infusions of the cannabinoid agonist WIN55,212-2, given 60 min before testing rats for memory retrieval, impaired such memory process only in animals trained under the high arousing condition. Furthermore we found that such effect was mediated by the activation of CB1 receptors, since a concurrent infusion of a non-impairing dose of the CB1 antagonist AM251 blocked the memory impairing effects of WIN55,212-2. In a second set of experiments, by employing the same experimental protocol, we examined whether the endogenous cannabinoids AEA and/or 2-AG could modulate spatial memory retrieval as well. To this aim the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor JZL184 were infused bilaterally into the hippocampus 60 min before the probe trial. We found that URB597 did not alter spatial memory retrieval performances in any of the two experimental conditions. Interestingly,

highly comparable with WIN55,212-2 effects, JZL184 impaired spatial memory retrieval only in rats trained at the lower, high-arousing, temperature *via* the activation of CB1 receptors. This experimental protocol also produced different biochemical effects on the cannabinoid system. The higher stressful experimental conditions induced an increase in hippocampal 2-AG levels, without altering hippocampal AEA levels, after the training and probe sessions. Consistently, immediately after testing rats for memory retrieval, we found an alteration in the binding affinity for MAGL in animals under the high arousing condition without observing any alteration in the hippocampal FAAH activity. Hippocampal CB1 receptors functionality analysis showed a decrease in maximal binding site density for both high aroused and low aroused rats.

Previous findings demonstrated that rats trained in a Morris water maze at 19 °C exhibited both better memory consolidation and retrieval than rats trained at 25 °C (Akirav *et al*, 2004; Akirav *et al*, 2001; Sandi *et al*, 1997). A more recent study reported that animals trained in a radial arm water maze task showed an inverted U-shape memory function according with the stressfulness of the experimental conditions employed (Salehi *et al*, 2010). Performance of rats trained at 19 °C was more accurate than that of animals trained at either higher (16 °C) or lower (25 °C) stress conditions (Salehi *et al*, 2010). It has also been shown that rats trained at 19 °C presented higher levels of corticosterone than rats trained at 16° C and lower than animals trained at 25 °C (Akirav *et al*, 2004; Akirav *et al*, 2001; Salehi *et al*, 2010; Sandi *et al*, 1997). Accordingly, our results showed a stronger memory consolidation, as indicated by shorter latencies to find the hidden platform during the second day of training, in rats in the high arousal conditions relative to low aroused animals. It is possible that the experimental conditions have elicited a different release of corticosterone depending on the level of stress induced by the two water temperatures and, subsequently, such hormone may have affected differentially memory consolidation mechanisms of the two groups of rats. Importantly, our results strongly support the current view that stress (and glucocorticoids) facilitates memory consolidation (de Quervain *et al*, 2009; Oitzl and de Kloet, 1992; Roozendaal *et al*, 2008; Roozendaal *et al*, 1999; Sandi *et al*, 2007; Sandi and Rose, 1994). Our findings fall in line with the proposed inverted U-shaped dose-response relationship between stress and memory performance (Lupien *et al*, 1997). Extreme low and high levels of stress and glucocorticoids impair consolidation, whereas moderate activation seems to be a prerequisite for

the long-term storage of information (Conrad *et al*, 1997; Diamond *et al*, 1992; Diamond *et al*, 1999; Flood *et al*, 1978; Liu *et al*, 1999; Oitzl *et al*, 1994). The differential behavioral performances exhibited by rats trained at different water temperatures might account for the first raising part of the inverted U-shaped curve. Consistently, high aroused rats, also showed a better memory retrieval of a strongly consolidated training, relative to low aroused rats.

Extensive cognitive and neurobiological research on animals, healthy human subjects, and amnesic patients indicates that the hippocampus is an important brain region involved in memory retrieval (Brun *et al*, 2002; Cabeza and Nyberg, 2000; Eldridge *et al*, 2000; Hirsch, 1974; Holt and Maren, 1999; Matus-Amat *et al*, 2004; Moser and Moser, 1998; Riedel *et al*, 1999; Schacter and Wagner, 1999; Squire *et al*, 2001) and is also a primary target of stress hormones (Reul and de Kloet, 1985). CB1 receptors are also abundantly expressed on hippocampal GABAergic terminals and, to a minor extent, glutamatergic terminals (Katona *et al*, 1999). Findings of several studies indicates that stress and glucocorticoids typically impair memory retrieval in both animal and human subjects (de Quervain *et al*, 2009; de Quervain *et al*, 1998; Roozendaal, 2000; Roozendaal *et al*, 2004a). We have recently shown that blockade of hippocampal CB1 receptors with local infusions of AM251 prevented the glucocorticoid-induced impairment of contextual fear memory retrieval thus indicating that endocannabinoid signaling plays an important role in regulating stress effects on this memory phase (Atsak *et al*, 2012). Moreover, we also found that intra-hippocampal infusions of WIN55,212–2 impaired the retrieval of contextual fear memory (Atsak *et al*, 2012). Here we showed that cannabinoid impairing effects on memory retrieval are strictly dependent on the level of emotional arousal associated to the experimental condition. Indeed we found that intra-hippocampal infusions of WIN55,212–2 in high aroused rats impaired spatial memory retrieval and such effect was mediated by the activation of CB1 receptors, since hippocampal infusion of AM251 blocked the WIN55,212–2 impairing effect. Conversely, the same pharmacological manipulation did not induce any effect on memory in low aroused rats. Accordingly with our findings, intra-hippocampal infusions of WIN55,212–2 have been reported to impair memory retrieval of high arousing test situations (Piri *et al*, 2011; Segev *et al*, 2011). We further found that intra-hippocampal infusions of the FAAH inhibitor URB597 did not alter memory performance in any of the two experimental conditions. Interestingly, highly

comparable with WIN55,212–2 effects, intra-hippocampal infusions of the 2-AG hydrolysis inhibitor JZL184 impaired memory retrieval *via* an indirect activation of CB1 receptors, only in high aroused rats. Taken together these findings demonstrated that hippocampal 2-AG, but not AEA, mediates memory retrieval impairment under high arousing situations. In accordance with these results, we previously showed that impairing effects on contextual fear memory retrieval induced by pretest corticosterone administration are mediated by an increase of hippocampal 2-AG (Atsak *et al*, 2012).

Furthermore, we found that the high stressful experimental condition, in not-treated rats, also induced an increase in hippocampal 2-AG, without altering AEA levels, 20 min after the training sessions and immediately after probe. These findings are consistent with previous evidence that stress and glucocorticoids rapidly alter endocannabinoid signaling in a variety of stress-responsive brain regions, including the hippocampus (Hill *et al*, 2010a; Hill and McEwen, 2010b). Although some controversy exists in the literature, stress has been shown to mobilize 2-AG while concurrently decreasing AEA levels in the hippocampus (Hill *et al*, 2010b; Patel and Hillard, 2008). Consistently with the increase in 2-AG levels, we also found that the high arousing training procedure at 19 °C did not alter FAAH activity but induced a decrease in MAGL affinity for its substrate 2-AG. CB1 receptor maximal binding site density decreased in both experimental groups. It is likely that the stress associated to the behavioral procedure *per se*, regardless of its level, had induced this change in hippocampal CB1 receptors. Although not fully in accordance with our results, it has previously been shown that chronic corticosterone administration in drinking water decreases CB1 receptor binding site density in the hippocampus, reduced AEA content, increased FAAH activity and significantly increased 2-AG concentrations within the hippocampus (Bowles *et al*, 2012). However, we employed a completely different experimental procedure associated to lower level of stress that might have induced only some of these previously reported alterations.

As previously highlighted, stress effects on memory follow an inverted U-shaped pattern, we therefore can speculate that the higher stressful conditions during trainings at 19 °C might have increased hippocampal 2-AG to optimal levels to ensure a stronger consolidation, thus promoting a better retrieval of spatial memory. In contrast to the consolidation phase, it is unlikely that glucocorticoids might have influenced a 1-min memory retrieval performance in vehicle treated

rats. However, it is more plausible that the arousal-induced noradrenergic activity, in concert with optimal levels of 2-AG, might have induced an enhancement in memory retrieval. Indeed, the hippocampus receives a dense input of noradrenergic terminals (Schroeter *et al*, 2000), and during emotionally arousing conditions norepinephrine is rapidly released in the brain (Gold and van Buskirk, 1978; McIntyre *et al*, 2002) typically returning to baseline levels within 10 min, but the glucocorticoid response is much slower (De Boer *et al*, 1990). Corroborating our findings, a recent clinical study showed that retrieval performance under stress is positively correlated with autonomic arousal, expressed as change in blood pressure, but unaffected by cortisol. Conversely, retrieval performance 25 min post stress was negatively correlated with the cortisol response to the stressor (Schonfeld *et al*, 2013).

Detrimental effects of glucocorticoids on memory retrieval of emotionally arousing experiences depend crucially on an interaction with arousal-induced noradrenergic activity (de Quervain *et al*, 2007; Roozendaal *et al*, 2003; Roozendaal *et al*, 2004b; Schutsky *et al*, 2011) and are mediated by hippocampal 2-AG (Atsak *et al*, 2012). We have recently shown that systemic injection of WIN55,212-2 increases corticosterone plasma levels when injected in rats trained in an object recognition task and never habituated to experimental arena (high aroused), while the same compound decreases plasma corticosterone levels in rats which were in a less aroused state because of an extensive prior habituation to the training context (Campolongo *et al*, 2013). In the present study, on the one hand, intra-hippocampal WIN55,212-2 administration may have induced the same increasing effects on plasma corticosterone levels and, further, by directly stimulating CB1 receptors, and interacting with the arousal-induced noradrenergic activity, may have impaired spatial memory retrieval only in high aroused rats. On the other hand, intra-hippocampal JZL184 administration, by further increasing 2-AG levels may have mimicked corticosterone increasing effects on hippocampal 2-AG (Atsak *et al*, 2012) which interacts with concurrent arousal-dependent increases in noradrenergic activity (Atsak *et al*, 2012), thus inducing detrimental effects on spatial memory retrieval observed only in rats trained and tested under the lower, high arousing, water temperature.

Collectively, the present findings indicate that the hippocampal endocannabinoid system plays a key role in mediating emotional arousal effects on spatial memory retrieval, shedding light on the neurobiological mechanism involved in the differential impact of stress on memory processes. Thus, the findings of the

present study may be relevant to develop new therapeutic strategies for psychiatric pathologies characterized by cognitive disturbances, such as post-traumatic stress disorder.

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CHAPTER 5

THE ENDOCANNABINOID SYSTEM: AN EMOTIONAL BUFFER IN THE MODULATION OF MEMORY FUNCTION

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Abstract

Extensive evidence indicates that endocannabinoids modulate cognitive processes in animal models and human subjects. However, the results of endocannabinoid system manipulations on cognition have been contradictory. As for anxiety behavior, a duality has indeed emerged with regard to cannabinoid effects on memory for emotional experiences. Here we summarize findings describing cannabinoid effects on memory acquisition, consolidation, retrieval and extinction. Additionally, we review findings showing how the endocannabinoid system modulates memory function differentially, depending on the level of stress and arousal associated with the experimental context. Based on the evidence reviewed here, we propose that the endocannabinoid system is an emotional buffer that moderates the effects of environmental context and stress on cognitive processes.

Introduction

Emerging evidence indicates that cannabinoid drugs can induce distinct and often opposite effects on anxiety, cognition, and several other behaviors, depending on stress level and the aversiveness of the context (Campolongo *et al*, 2012; Haller *et al*, 2009; Szuster *et al*, 1988; Zanettini *et al*, 2011). Although cannabinoid signaling has been demonstrated to influence memory processing (Campolongo *et al*, 2009b; Marsicano *et al*, 2002), it is difficult to define its exact role because, regardless of the pharmacodynamic properties of the drug, both impairing and enhancing effects have been reported with cannabinoid drug administration. Although such discrepancies are not unusual in memory research, the factors contributing to these conflicting findings remain poorly understood.

In this review, we begin with a summary of the differing memory modulatory effects of endocannabinoids reported in the literature. We then discuss in detail the biphasic/opposite effects induced by cannabinoid drugs, including evidence that such effects may be strongly dependent on the aversiveness of environmental context and on the level of stress at the time of drug administration and/or training. Finally, with the ultimate aim of developing an explanation of the apparent discrepancies among studies of cannabinoid effects on memory function, we propose hypotheses to explain the observed dual/opposing effects of cannabinoids on emotional memory functions.

The endocannabinoid system

The discovery of the main psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), led to the identification of the endogenous endocannabinoid system (Gaoni and Mechoulam, 1964). The endocannabinoid system is a lipid signaling system in the brain that begins to exhibit functional activity early in brain development by way of modulating neurotransmitter release, pre- and postnatally (Campolongo *et al*, 2009c; Campolongo *et al*, 2011; Fernandez-Ruiz *et al*, 2000; Fride, 2004; Harkany *et al*, 2007; Trezza *et al*, 2008; Trezza *et al*, 2012). Although many molecular targets of the endocannabinoid system have been described, the primary targets of cannabinoid compounds are the type 1 and type 2 cannabinoid receptors (CB1 and CB2, respectively) (Devane *et al*, 1988; Herkenham *et al*, 1990; Matsuda *et al*, 1990).

The two major endogenous ligands for the CB1 and CB2 receptors are *N*-arachidonoyl ethanolamine (anandamide, AEA) (Devane *et al*, 1992) and 2-arachidonoyl glycerol (2-AG) (Sugiura *et al*, 1995). AEA acts as a partial agonist of CB1 and CB2 receptors (Pertwee, 2010), whereas 2-AG is full agonist of these receptors (Stella *et al*, 1997). Unlike classical neurotransmitters, endocannabinoids are not stored in presynaptic vesicles, but rather are synthesized postsynaptically from lipid membrane precursor molecules in an

activity-dependent manner (Kano *et al*, 2009). Once released from the postsynaptic membrane into the synaptic cleft, they travel backward to bind cannabinoid receptors expressed on presynaptic terminals. Activation of CB1 receptors inhibits neurotransmitter release by modulating several ion channels and kinases (Kano *et al*, 2009; Turu and Hunyady, 2010). Following receptor activation, AEA and 2-AG are deactivated by a still poorly defined uptake process involving a transporter mechanism (Fu *et al*, 2011; Hillard *et al*, 1997). Subsequently, they are metabolized mainly by their respective degradative enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Kano *et al*, 2009).

CB1 receptors represent the most abundant class of G-protein-coupled receptors in the central nervous system, and are also present in a variety of peripheral tissues. They couple with both G_i and G_o proteins, which inhibit adenylyl cyclase activity, activate potassium channels, and inhibit voltage-gated calcium channels (Howlett *et al*, 2002). CB1 receptors are expressed abundantly in major structures of the limbic system, including the hippocampus and basolateral complex of the amygdala (BLA), as well as in the prefrontal cortex (PFC), which is closely linked with limbic structures (McPartland *et al*, 2007); low levels of CB1 mRNA have also been detected in the central nucleus of the amygdala (CeA) (Kamprath *et al*, 2010; Marsicano and Lutz, 1999; Matsuda *et al*, 1993). Within these limbic regions, the CB1 receptor is expressed at very high levels in cholecystokinin-positive GABAergic interneurons (Azad *et al*, 2008; Marsicano *et al*, 1999; Morozov *et al*, 2009) and at moderate levels in glutamatergic terminals (Kano *et al*, 2009; Kawamura *et al*, 2006; Monory *et al*, 2006). The CB1 receptor has also been detected on serotonergic, noradrenergic, and dopaminergic terminals (Haring *et al*, 2007; Hermann *et al*, 2002; Oropeza *et al*, 2007).
The CB2 receptor is a $G_{i/o}$ protein-coupled receptor (Howlett *et al*, 2002). CB2 receptors are located mostly in the periphery on immunological tissues. They were confirmed only recently by immunohistochemical analyses to be expressed by neurons and glia in diverse rat brain areas, including the cerebellum and hippocampus (Onaivi *et al*, 2006; Van Sickle *et al*, 2005).

Studies examining the functions of endocannabinoid signaling in the limbic system have shown that CB1 receptors play a key role in modulating synaptic transmission (Katona *et al*, 2001; Tan *et al*, 2011) and neuronal firing (Pistis *et al*, 2004). Furthermore, growing evidence indicates that endocannabinoids play a key role in modulating emotional memory processes (Atsak *et al*, 2012b; Campolongo *et al*, 2009b; Ganon-Elazar and Akirav, 2009; Marsicano and Lafenetre, 2009; Marsicano *et al*, 2002; Tan *et al*, 2011; Wotjak, 2005). In the succeeding sections, we provide a review of findings from studies that examined cannabinoid effects on emotional memory function, focusing especially on the functional relationship between endocannabinoids and glucocorticoids in modulating cognitive

processes. Subsequently, we discuss how stress and arousal state may modulate endocannabinoid effects on memory.

Modulation of memory for emotional experiences

Emotional learning is extremely important for the survival of an individual; indeed life events of positive and negative valence typically leave lasting and vivid memories due to arousal and stress hormone effects on memory consolidation (McGaugh, 2000). Emotionality describes a highly complex repertoire of behaviors triggered by various environmental stimuli. The regulation of emotional responses under different environmental conditions is essential for mental health and requires fine-tuned neurotransmitter release processes as well as functional neuronal circuits (Gold, 2004; McEwen, 2012; McGaugh, 2000). During emotionally arousing situations, stress hormones are released from the adrenal medulla (epinephrine) and cortex (corticosterone [CORT] in rats, cortisol in humans) into the bloodstream. These systemic stress hormones stimulate the vagus nerve in the periphery, thereby activating the nucleus of tractus solitarius (NTS) in the brainstem, which releases memory modulatory norepinephrine into limbic brain structures (McGaugh and Roozendaal, 2002).

Additionally, glucocorticoid hormones, which are highly lipophilic, readily enter the brain where they bind mineralocorticoid receptors (MRs) with high affinity and glucocorticoid receptors (GRs) with low affinity. Thus, under basal conditions, only MRs are occupied, but during and immediately after a stressful experience, both MRs and GRs are bound by glucocorticoids (Reul and de Kloet, 1985). Extensive evidence indicates that stress hormones, in concert with several other stress-activated systems, mediate the selective enhancement of consolidation of memory for emotionally significant experiences (de Kloet *et al*, 1999; Joels and Baram, 2009; Oitzl and de Kloet, 1992; Roozendaal, 2000; Sandi and Rose, 1994). Conversely, glucocorticoids typically impair memory retrieval and working memory during emotionally arousing test situations (de Quervain *et al*, 2009; de Quervain *et al*, 1998; Roozendaal, 2000; Roozendaal *et al*, 2004).

The neural circuitry underlying emotionality is considerably complex, but broadly consists of subcortical limbic structures, such as the amygdala, hippocampus, ventral striatum, and thalamus, as well as cortical structures, including the anterior cingulate cortex and medial and orbital regions of the PFC (Price and Drevets, 2010). This corticolimbic circuit interacts with visceral autonomic centers in the hypothalamus and brain stem to regulate emotional expression and to modulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Price *et al*, 2010). In this assembly, the amygdala represents a key region for the association of environmental information with emotional significance. Although the acquisition of emotional salience by external stimuli has been studied most extensively in relation to fear and anxiety responses, the

amygdala has also been shown to be important for the processing of positive emotions, such as in stimulus-reward learning (Aggleton, 1993; Baxter and Murray, 2002; Davis *et al*, 1994; Pape and Pare, 2010).

In particular, considerable evidence indicates not only that stressors increase neuronal activity in the BLA (Pelletier *et al*, 2005), but also that emotional memory modulation requires activation of the BLA specifically. For example, lesions of the BLA, but not the CeA, block the memory enhancing effects of systemic GR activation on inhibitory avoidance retention (Roozendaal and McGaugh, 1996). Furthermore, posttraining infusion of norepinephrine or a β-adrenoceptor agonist into the BLA enhances memory of training on several learning tasks (Ferry and McGaugh, 1999; Hatfield *et al*, 1999; LaLumiere *et al*, 2003; Roozendaal *et al*, 2008). In contrast, attenuation of noradrenergic signaling by infusion of a β-adrenoceptor antagonist (propranolol or atenolol) into the BLA, but not into the neighboring CeA, has been shown to block the memory enhancement induced by systemic or intra-BLA administration of a GR agonist (Quirarte *et al*, 1997; Roozendaal *et al*, 2002). Considerable evidence developed in rodent studies indicates that glucocorticoid-induced enhancement of memory consolidation depends upon an interaction with noradrenergic activation within the BLA (Roozendaal *et al*, 2009). Importantly, a recent clinical study corroborated this model by showing that the amygdala is also an important locus of glucocorticoid-norepinephrine interactions in the enhancement of memory for emotionally salient information in humans (van Stegeren *et al*, 2010). Indeed, a convergence of five decades of research now points to the amygdala, especially the BLA, as a critical structure in the acquisition and retention of lasting memories of emotional experiences (McGaugh, 2000; Roozendaal *et al*, 2009).

Cannabinoid modulation of memory for emotional experiences

Recent evidence suggests that endocannabinoid signaling may be a key system in the regulation of synaptic efficacy within and between amygdaloid subnuclei (Ramikie and Patel, 2012). Notably, glucocorticoids regulate the endocannabinoid response, which in turn, modulates glucocorticoid secretion through both local and distal regulation of HPA axis activity (Hill and McEwen, 2010b; Hill *et al*, 2010c; Steiner and Wotjak, 2008b). Indeed, endocannabinoid activity is necessary for some central effects of glucocorticoids (Barna *et al*, 2004; Weidenfeld *et al*, 1994).

It has been demonstrated that an increase in glucocorticoid levels leads to a concomitant increase in endocannabinoid levels in the hypothalamus (Di *et al*, 2003). Endocannabinoids then act retrogradely to inhibit glutamate release in the paraventricular nucleus and suppress HPA axis activity (Di *et al*, 2003; Di *et al*, 2005). Conversely, endocannabinoid content in limbic brain regions, which can augment or terminate stress responses, can itself be modulated by stress or

exogenous glucocorticoid administration (Hill *et al*, 2010b). Additionally, Morrish and colleagues found that the endocannabinoid system mediates stress responses and emotional homeostasis by targeting noradrenergic circuits (Morrish *et al*, 2009).

The supposition that the endocannabinoid system may mediate a noradregeneric-modulatory role is supported by anatomic and physiological evidence. Autoradiography and immunohistochemistry experiments have demonstrated moderate CB1 receptor expression in the principal noradrenergic nuclei, namely the locus coeruleus (LC) and the NTS, and reverse transcription polymerase chain reaction experiments have confirmed local CB1 transcription in these regions (Derbenev *et al*, 2004; Herkenham *et al*, 1991; Jelsing *et al*, 2008; Mailleux and Vanderhaeghen, 1992; Matsuda *et al*, 1993). Systemic administration of the synthetic cannabinoid receptor agonist WIN55212-2 (WIN) increases noradrenergic release in the PFC (Oropeza *et al*, 2005), and intravenous injection of WIN or Δ^9-THC, increases the firing rate of LC noradrenergic neurons in a manner that is dose dependent and can be blocked by the cannabinoid receptor antagonist rimonabant (Muntoni *et al*, 2006). These findings suggest a role for the cannabinoid system in at least basic brain activities regulated by norepinephrine, such as arousal and wakefulness (Berridge *et al*, 2012).

More recent evidence suggests that the cannabinoid system may also play a key role in higher level noradrenergic functions, such as regulation of emotional states and memory processes (Akirav, 2011; Campolongo *et al*, 2009b; Kano *et al*, 2009; Marsicano *et al*, 2009; Wotjak, 2005). However, the literature regarding cannabinoid effects on cognition remains contradictory. Human cannabis users have been reported to show impairments in aspects of executive functioning such as planning, working memory, and mental flexibility (Verdejo-Garcia *et al*, 2006). Cannabis abusers also show short-term memory deficits (Ranganathan and D'Souza, 2006; Riedel and Davies, 2005). However, Fisk and Montgomery (2008) found no evidence of cannabis-related deficits in executive component processes or associative learning. Moreover, the acute effects of cannabis need to be distinguished from the effects of chronic use. Acutely, cannabis has well-known psychoactive effects, can impair coordination, and may produce feelings of anxiety or paranoia. Meanwhile, chronic use of cannabis has been reported to induce mood disturbances, exacerbate psychiatric disorders in vulnerable people, impair cognition, and to increase the risk of cardiovascular and respiratory diseases (for a detailed review see (Karila *et al*, 2013)). Although new evidence continues to become available, it would be premature to draw any strong conclusions from clinical studies of cannabis users/abusers now due to the widely differing methodologies and participant selection strategies used, especially in terms of poly-drug abuse and pre-existing cognitive and emotional criteria (Ranganathan *et al*, 2006). Therefore, animal studies remain critical to elucidating

the neural underpinnings of cannabinoid effects on cognition, particularly with respect to cannabinoid effects on memory for emotional experiences.

Cannabinoid effects on different memory phases

As will become clear, even basic research studies in this field are producing some controversial and difficult to resolve outcomes. In the following paragraphs, we summarize some basic research findings of cannabinoid effects on different phases of memory processes with the intent of disentangling which effects involve influences on particular stages of memory processing. Cannabinoid effects on memory acquisition, consolidation, retrieval, and extinction are summarized in Tables 1–4, respectively.

Cannabinoid effects on memory acquisition

There is general agreement about the observation that activation of the endocannabinoid system impairs memory acquisition (Table 1). Notably, systemic administration of a cannabinoid receptor agonist (i.e. Δ^9-THC or WIN) before training impairs acquisition of water maze, contextual fear, and object recognition tasks in rodents (Da and Takahashi, 2002; Lichtman *et al*, 1995; Pamplona and Takahashi, 2006b). Similarly, indirect cannabinoid receptor agonism impairs memory acquisition in a recognition memory task (Campolongo et al., 2012) and in the inhibitory avoidance task (Campolongo *et al*, 2012; Mazzola *et al*, 2009). However, as highlighted in Table 1, systemic administration of the cannabinoid receptor antagonist rimonabant has also been reported to impair acquisition in a spatial memory task (Robinson *et al*, 2008).

Local infusions of cannabinoid compounds within the same brain region have provided more consistent results, though comparing the results across brain areas remains difficult to explain. For example, pretraining activation of CB1 receptors in the hippocampus has consistently been shown to impair spatial learning (Abush and Akirav, 2010; Egashira *et al*, 2002; Lichtman *et al*, 1995; Wegener *et al*, 2008), although pretraining blockade of CB1 receptor transmission in the BLA also impaired olfactory fear conditioning (Tan *et al*, 2011). Drawing conclusions from studies involving pretraining drug administration is extremely challenging given that such treatments necessarily affect diverse processes (McGaugh, 1966). Moreover, since cannabinoid compounds affect motivational and sensorimotor processes (Economidou *et al*, 2007; Solinas and Goldberg, 2005; Steiner *et al*, 1999; Zimmer *et al*, 1999), it is difficult to discriminate between purely cognitive effects and confounding variables (e.g. alteration in pain sensitivity and/or locomotor activity and/or motivation) following pretraining cannabinoid administration. Therefore, it remains to be resolved whether cannabinoids affect cognition *per se* during learning rather than other non-specific factors.

Cannabinoid effects on memory consolidation

Conflicting data have been reported regarding cannabinoid effects on memory consolidation (Table 2). Drugs can be administered after a learning event to isolate the consolidation phase of memory and exclude influences on acquisition or any sensory, motor, or motivational processes that may influence learning indirectly (McGaugh, 1966). Systemic posttraining administration of cannabinoid receptor agonists impairs memory consolidation (Mackowiak *et al*, 2009; Yim *et al*, 2008), while systemic posttraining injection of cannabinoid receptor antagonists improves it (Wise *et al*, 2008; Wolff and Leander, 2003). However, there are concerns related to non-specificity in experiments using systemic agonist manipulations. Drugs that inhibit endocannabinoid degradation can be employed to avoid such potentially confounding effects. For example, when Busquets-Garcia and colleagues tested the effects of systemic posttraining administration of the FAAH inhibitor URB597, which increases AEA levels only in those brain regions where it is released endogenously, they observed impaired memory consolidation in an object recognition task (Busquets-Garcia *et al*, 2011).

Conflicting data have been reported concerning the effects on memory consolidation of infusing cannabinoid drugs locally into discrete brain regions. Posttraining intra-hippocampal administration of the synthetic cannabinoid receptor agonist WIN (0.25-10 μg/rat) has been reported to impair memory consolidation of several behavioral tasks (i.e. step-through inhibitory avoidance, Morris water maze) (Jamali-Raeufy *et al*, 2011; Yim *et al*, 2008; Zarrindast *et al*, 2011). However, other authors reported enhancing effects of AEA in the hippocampus (0.17 ng/side) (De Oliveira Alvares *et al*, 2008) and of WIN in the BLA (50 ng/side) (Campolongo *et al*, 2009b). Moreover, cannabinoid receptor antagonists have been found to yield memory impairing effects similar to those induced by cannabinoid receptor antagonism described above (Jamali-Raeufy *et al*, 2011; Yim *et al*, 2008; Zarrindast *et al*, 2011). In particular, it has been shown that intra-hippocampal (de Oliveira Alvares *et al*, 2005) administration of the CB1 receptor antagonist AM251 impairs memory consolidation in an aversive hippocampus-dependent task (de Oliveira Alvares *et al*, 2005). Differences in handling procedures, experimental conditions, behavioral task, doses, and the drug administered may account for the diversity of findings reported (Table 2). Indeed WIN is less selective than the endogenous ligand AEA (Howlett *et al*, 2002) and, when administered at high doses in discrete brain regions, could induce broader effects, complicating interpretation. However, there are other intriguing possibilities that should be explored. In particular, given the robust effects of glucocorticoids, norepinephrine, and other neurotransmitters including acetylcholine and dopamine in limbic structures on memory consolidation (McGaugh, 2000), it could be that factors related to arousal, stress, and emotional

state at the time of training may influence cannabinoid effects on memory. This issue is discussed further in section 5.

Table 1. Cannabinoid effects on memory acquisistion in rodents.

Drug	Dose	Administration	Animals	Paradigm	Effect	Reference
CB1/CB2 receptor agonists						
WIN	2.5-5 mg/kg	i.p.	Wistar rats	CFC	Impairing	(Pamplona *et al*, 2006b)
	1.2 mg/kg	i.p.	Sprague-Dawley rats	Object recognition	Impairing	(Schneider *et al*, 2008)
	5 µg/side	Intra-CA1	Sprague-Dawley rats	MWM	Impairing	(Abush *et al*, 2010)
Δ^9-THC	10 mg/kg	i.p.	FAAH -/-, +/+ mice	MWM	Impairing	(Wise *et al*, 2012)
	20 µg/side	Intra- DH-VH-DMT	Wistar rats	Eight-arm radial maze	Impairing	(Egashira *et al*, 2002)
CB1 antagonists (and inverse agonists)						
AM251	50–500 ng/side	Intra-BLA	Sprague-Dawley rats	Olfactory fear conditioning	Impairing	(Tan *et al*, 2011)
Rimonabant	3 mg/kg	i.p	Sprague-Dawley rats	Radial arm maze	Enhancing	(Lichtman, 2000)
	3 mg/kg	i.p.	Lister Hooded rats	MWM	Impairing	(Robinson *et al*, 2008)
AM281	2.5 mg/kg	i.p.	C57BL/6J mice	CFC	Enhancing	(Lin *et al*, 2011)
	0.05 µg/rat	Intra-CA1	C57BL/6J mice	CFC	Enhancing	(Lin *et al*, 2011)
Indirect agonists						
AM404 (AEA uptake inhibitor)	0.5–1 mg/kg	i.p.	Wistar rats	Spatial open field	Impairing	(Campolongo *et al*, 2012)
	1 µg/rat	Intra-CA1	C57BL/6J mice	CFC	Impairing	(Lin *et al*, 2011)
URB597 (FAAH inhibitor)	0.1–1.0 mg/kg	i.p.	Sprague-Dawley rats	IA	Impairing	(Mazzola *et al*, 2009)
JZL195 (FAAH and MAGL inhibitor)	20 mg/kg	i.p.	FAAH -/- mice	MWM	Impairing	(Wise *et al*, 2012)
	20 mg/kg	i.p.	FAAH +/+ mice	MWM	Impairing	(Wise *et al*, 2012)
JZL184 (MAGL inhibitor)	20–40 mg/kg	i.p.	FAAH -/- mice	MWM	Impairing	(Wise *et al*, 2012)
	40 mg/kg	i.p.	FAAH +/+ mice	MWM	Impairing	(Wise *et al*, 2012)

AEA, anandamide; i.p., intraperitoneal; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; CFC, contextual fear conditioning; MWM, Morris water maze; IA, step-through inhibitory avoidance; BLA, basolateral complex of the amygdala; CeA, central amygdala; DH, dorsal hippocampus; VH, ventral hippocampus; DMT, dorsomedial thalamus nucleus.

Table 2. Cannabinoid effects on memory consolidation in rodents.

Drug	Dose	Administration	Animals	Paradigm	Effect	Reference
CB1/CB2 receptor agonists						
AEA	0.17 ng/side	Intra-CA1	Wistar rats	IA	Enhancing	(De Oliveira Alvares *et al*, 2008)
HU-210	0.1 mg/kg	i.p.	Wistar rats	CFC	Impairing	(Mackowiak *et al*, 2009)
WIN	1-3 mg/kg	i.p.	Long-Evans rats	MWM	Impairing	(Yim *et al*, 2008)
	0.3 mg/kg	i.p.	Sprague-Dawley rats	HA-object recognition	Enhancing	(Campolongo *et al*, 2013)
	50 ng/side	Intra-BLA	Sprague-Dawley rats	IA	Enhancing	(Campolongo *et al*, 2009b)
	0.1–0.25 µg/rat	Intra-CeA	Wistar rats	IA	Impairing	(Zarrindast *et al*, 2012)
	0.25–0.5 µg/rat	Intra-CA1	Wistar rats	IA	Impairing	(Moshfegh *et al*, 2011)
	0.1–0.5 µg/rat	Intra-CA1	Wistar rats	IA	Impairing	(Nasehi *et al*, 2009)
	0.25-0.5 µg/rat	Intra-CA1	Wistar rats	IA	Impairing	(Jamali-Raeufy *et al*, 2011)
	10 nmol/side	Intra-CA1	Wistar rats	Object recognition	Impairing	(Clarke *et al*, 2008)
CB1 antagonists (and inverse agonists)						
AM251	0.28 ng/side	Intra-BLA	Sprague-Dawley rats	IA	Impairing	(Campolongo *et al*, 2009b)
	0.28 ng/side	Intra-BLA	Wistar rats	CFC	Impairing	(Bucherelli *et al*, 2006)
	5.5 ng/side	Intra-CA1	Wistar rats	IA	Impairing	(de Oliveira Alvares *et al*, 2005)
Rimonabant	1 mg/kg	i.p.	Sprague-Dawley rats	Eight-arm radial maze	Enhancing	(Wolff *et al*, 2003)
	1 mg/kg	i.p.	Swiss albino mice	Elevated T-maze	Enhancing	(Takahashi *et al*, 2005)
CE	0.1 mg/kg	i.p.	Sprague-Dawley rats	Radial-arm maze	Enhancing	(Wise *et al*, 2008)
Indirect agonist						
URB597 (FAAH inhibitor)	0.3–1 mg/kg	i.p.	Swiss albino mice	Object recognition	Impairing	(Busquets-Garcia *et al*, 2011)
	1 mg/kg	i.p.	Swiss albino mice	Context recognition	Impairing	(Busquets-Garcia *et al*, 2011)

AEA, anandamide; i.p., intraperitoneal; FAAH, fatty acid amide hydrolase; CFC, contextual fear conditioning; MWM, Morris water maze; IA, step-through inhibitory avoidance; HA, high arousal; BLA, basolateral complex of the amygdala; CeA, central amygdala.

Cannabinoid effects on memory retrieval

Although the literature related to cannabinoid effects on retrieval is fairly limited, no controversy exists with regards to cannabinoid effects on memory retrieval (Table 3). Detrimental effects of cannabinoid receptor agonism on memory

Table 3. Cannabinoid effects on memory retrieval in rodents.

Drug	Dose	Administration	Animals	Paradigm	Effect	Reference
CB1/CB2 receptor agonists						
WIN	0.3 mg/kg	i.p.	Sprague-Dawley rats	HA-object recognition	Impairing	(Campolongo *et al*, 2013)
	0.3 mg/kg	i.p.	Sprague-Dawley rats	LA-object recognition	Enhancing	(Campolongo *et al*, 2013)
	10–30 ng/side	Intra-CA1	Sprague-Dawley rats	CFC	Impairing	(Atsak *et al*, 2012a)
	0.25–0.5 µg/rat	Intra-CA1	Wistar rats	IA	Impairing	(Piri *et al*, 2011)
	5 µg/side	Intra-VSub	Sprague-Dawley rats	CFC	Impairing	(Segev *et al*, 2011)
Δ^9-THC	5.6 mg/kg	i.p	Sprague-Dawley rats	Radial arm maze	Impairing	(Wise *et al*, 2009)
	6 mg/kg	i.p.	Wistar rats	Eight-arm radial maze	Impairing	(Mishima *et al*, 2001)
	10 mg/kg	i.p.	C57BL/6J mice	MWM	Impairing	(Niyuhire *et al*, 2007a)
	10 mg/kg	i.p.	Wistar rats	IA	Impairing	(Mishima *et al*, 2001)

I.p., intraperitoneal; CFC, contextual fear conditioning; MWM, Morris water maze; IA, step-through inhibitory avoidance; VSub, ventral subiculum; HA, highly arousal; LA, low arousal.

retrieval have been documented when administered either systemically (Mishima *et al*, 2001; Niyuhire *et al*, 2007a) or in discrete brain areas (Atsak *et al*, 2012a; Piri and Zarrindast, 2011; Segev and Akirav, 2011). In particular, it has been demonstrated that systemic administration of Δ^9-THC impairs memory retrieval in a Morris water maze task (Niyuhire *et al*, 2007a) and a step-through inhibitory avoidance task (Mishima *et al*, 2001). In line with these findings, cannabinoid agonists infused directly into the hippocampus impair aversive memory retrieval (Atsak *et al*, 2012a; Piri *et al*, 2011; Segev *et al*, 2011). Interestingly, intra-BLA infusions of the same drugs did not affect memory retrieval (Segev *et al*, 2011). Taken together, the preclinical evidence reported above, although still sparse,

suggests that cannabinoids induce impairing effects on memory retrieval, at least under the experimental conditions investigated thus far (Table 3).

Cannabinoid effects on memory extinction

There is a strong consensus in the memory extinction literature that the endocannabinoid system is a key modulator in the facilitation of memory extinction (Table 4). Inhibition of endocannabinoid transmission robustly inhibits extinction of fear conditioning (Marsicano *et al*, 2002; Pamplona *et al*, 2006a; Suzuki *et al*, 2004). Conversely, stimulation of endocannabinoid signaling

Table 4. Cannabinoid effects on memory extinction in rodents.

Drug	Dose	Administration	Animals	Paradigm	Effect	Reference
CB1/CB2 receptor agonists						
WIN	0.25 mg/kg	i.p.	Wistar rats	CFC	Enhancing	(Pamplona *et al*, 2006a)
	5 µg/side	Intra-CA1	Sprague-Dawley rats	IA	Enhancing	(Abush *et al*, 2010)
CB1 antagonists (and inverse agonists)						
Rimonabant	1.5–5 mg/kg	i.p	Sprague-Dawley rats	FPS	Impairing	(Chhatwal *et al*, 2005)
	3 mg/kg	i.p.	C57BL/6J mice	Auditory fear conditioning	Impairing	(Marsicano *et al*, 2002)
	3–10 mg/kg	i.p.	C57BL/6J mice	CFC	Impairing	(Suzuki *et al*, 2004)
	3 mg/kg	i.p.	C57BL/6J mice	MWM	Impairing	(Varvel *et al*, 2005)
	3 mg/kg	i.p.	C57BL/6J mice	IA	Impairing	(Niyuhire *et al*, 2007b)
Indirect agonists						
AM404 (AEA uptake inhibitor)	10 mg/kg	i.p	Sprague-Dawley rats	FPS	Enhancing	(Chhatwal *et al*, 2005)
	1 µg/rat	i.c.v.	Wistar rats	CFC	Enhancing	(Bitencourt *et al*, 2008)
OL-135 (FAAH inhibitor)	30 mg/kg	i.p.	C57BL/6J mice	MWM	Enhancing	(Varvel *et al*, 2007)

I.p., intraperitoneal; i.c.v., intracerebroventricular; FAAH, fatty acid amide hydrolase; CFC, contextual fear conditioning; MWM, Morris water maze; IA, step-through inhibitory avoidance; FPS, fear-potentiaed startle.

accelerates fear extinction (Barad *et al*, 2006; Chhatwal *et al*, 2005; Pamplona *et al*, 2006a; Suzuki *et al*, 2004). Similarly, in a Morris water maze task, Varvel et al. (2005; 2007) found that the inverse CB1 receptor agonist rimonabant or genetic CB1 receptor disruption impaired extinction, whereas Δ^9-THC did not affect extinction (Varvel *et al*, 2005; Varvel *et al*, 2007). Interestingly, Niyuhire and co-workers (2007b) reported that rimonabant administration disrupted extinction significantly in two different aversively motivated behavioral tasks (e.g., conditioned freezing and inhibitory avoidance) but failed to affect extinction in an appetitively motivated operant conditioning task (Niyuhire *et al*, 2007b). Thus, the evidence obtained to date suggests that activation of the cannabinoid system facilitates aversive memory extinction.

Role of emotional arousal in influencing cannabinoid effects on memory

Discrepant findings have been reported concerning the role of the endocannabinoid system in the modulation of cognitive processes, especially with regard to memory acquisition and consolidation. Such reports are very reminiscent of the dual effects on emotionality reported by cannabis users. That is, although cannabis consumption is commonly associated with euphoria and contentment (Velez and Ungemack, 1989), some people report experiencing anxiety, dysphoria, and a depressive mood with cannabis consumption (Reilly *et al*, 1998). Clinical studies have indicated that the context of cannabinoid consumption—that is, the nature of both one's physical state and social setting—has a direct influence on emotionality (Del Porto and Masur, 1984; Zinberg, 1984), even though clinical studies are less controlled in terms of dosage, poli-drug abuse, and context. Biphasic effects of cannabinoid consumption on anxiety-related behavior have also been reported in preclinical studies (for a detailed review see (Micale *et al*, 2013). The indirect cannabinoid receptor agonists URB597 and AM404, which increase AEA levels in the synaptic cleft, have been reported to exert both anxiolytic- and antidepressant-like effects in rodents (Bortolato *et al*, 2006; Gobbi *et al*, 2005; Kathuria *et al*, 2003). Haller and co-workers (2009) showed that URB597 does not reduce anxiety when a behavioral task (i.e. elevated plus maze) is performed under mildly aversive conditions (e.g., in a familiar room or under low light) (Haller *et al*, 2009). Sciolino et al. (2011) found that an increase in 2-AG levels, induced by administration of the MAGL inhibitor JZL184, induced an anxiolytic-like effect in the same behavioral task under highly aversive conditions (i.e. bright light), but had no detectable effect on anxiety under low-stress conditions (Sciolino *et al*, 2011). Likewise, Campos et al. (2010) reported that the cannabinoid uptake inhibitor AM404 was anxiolytic in the elevated plus maze test in rats previously exposed to a 2-h restraint stress, but anxiogenic in rats that were not previously stressed (Campos *et al*, 2010). Thus, it may be that stress conditions during the test, as well as housing and handling

procedures prior to the test, influence cannabinoid effects on emotionality. A similar scenario is now emerging with regards to cannabinoid effects on memory. (Herkenham *et al*, 1991; Tsou *et al*, 1998). In the following sections, we describe evidence demonstrating how cannabinoid effects on memory may depend on the level of stress associated with an experimental context (i.e. footshock intensity in the inhibitory avoidance task), previous stress experiences completely unrelated to the task, and/or a combination of these two factors.

Context-induced stress shapes cannabinoid modulation of memory

The cannabinoid system may have a particularly important role in the control of neuronal responses to environmental challenges. This notion is consistent with the observation that CB1 receptors are expressed abundantly in limbic structures. It is possible to speculate that the endocannabinoid system may shape how environmental stimuli affect emotional responses, rather than producing an overall aspecific effect on memory. This putative context-dependence may help to explain apparently conflicting data obtained with different training and drug administration paradigms.

To investigate the importance of emotionality in cannabinoid effects on memory function, we compared the effects of cannabinoid receptor activation on novel object recognition in high arousal (HA) versus low-arousal (LA) conditions. Briefly, in the HA condition, the rats were not handled and the task was performed under bright light in an empty arena. In the LA condition, the rats were habituated to the experimenter through daily handling (1 min per day for 1 week) and the task was performed under dim red light in an arena in which the ground was covered with familiar bedding. Animals were administered the endocannabinoid transport inhibitor AM404 30 min before commencing the novel object recognition task. The behavioral paradigm consisted of six consecutive 5-min sessions, separated by 3-min intervals, wherein one familiar object was replaced with a new object in the last session, which served as the test trial. We found that exogenous enhancement of endocannabinoid signaling impaired novel object recognition in rats tested in the HA condition, but had no effect in rats tested in the LA condition (Campolongo *et al*, 2012). This study demonstrated for the first time that acute effects of cannabinoid agonism on memory function are influenced by the stress state of the subject. Because this experiment employed a pretraining drug administration, we cannot exclude the possibility that the memory performance effect observed could have been due, even in part, to confounding variables associated with a pretraining manipulation.

In a subsequent study, we employed a previously validated, modified version of the object recognition task that enabled us to administer the drug immediately after training to isolate the effect of the drug on memory consolidation. We followed a previously described procedure to induce two different levels of

arousal during the object recognition task (Okuda *et al*, 2004). One group of rats received extensive prior habituation to the training apparatus (in the absence of any objects), while a second group was not exposed to the experimental apparatus until training. Animals were injected with the cannabinoid receptor agonist WIN immediately after a single 3-min training trial and then tested for memory retention 1 h or 24 h later. As shown in Figure 1A and B, WIN, administered immediately after object recognition training, impaired short-term (1-h) retention performance in rats not habituated to the experimental context. The same dose of WIN enhanced short-term memory in rats that had been habituated to the experimental context (Campolongo *et al*, 2013). Meanwhile, WIN enhanced long-term (24-h) retention in non-habituated rats, but had no effect on long-term memory in habituated rats (Fig. 1C and D). This experience-dependent cannabinoid effect on memory is highly comparable to the glucocorticoid effects described by Okuda et al. (2004) and Roozendaal et al. (2006). That is, glucocorticoid compounds administered after object recognition training enhanced memory consolidation in non-habituated rats (a relatively higher stress condition) but not in habituated rats (a relatively lower stress condition (Okuda *et al*, 2004; Roozendaal *et al*, 2006b). Moreover, posttraining exposure to an out-of-context stressor (i.e. elevated platform) after object recognition training has been reported to enhance long-term memory only in rats not previously habituated to the experimental apparatus (Maroun and Akirav, 2008).

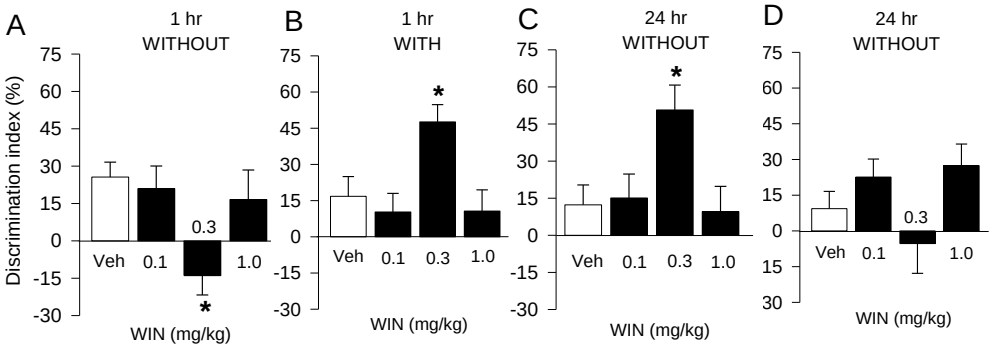

Figure 1. Effects of the CB receptor agonist WIN on short- and long-term retention of object recognition training are influenced by the level of training-associated emotional arousal. Rats were either habituated for 7 d (WITH) or not habituated (WITHOUT) to the training context. On day 8, they were given a 3-min training trial during which they could freely explore two identical objects, training was followed by a systemic administration of WIN (0.1, 0.3 or 1 mg/kg, i.p.). Retention was tested 1 h or 24 h later. Data represent discrimination index (%) at the retention trial, expressed as mean ± SEM. The

discrimination index was calculated as the difference in the time spent exploring the novel and the familiar object, expressed as a ratio of the total time spent exploring both objects. Posttraining WIN impaired 1-h object recognition performance of non-habituated rats (A) but enhanced performance of habituated rats (B). In contrast, posttraining administration of WIN, at a dose that impaired 1-h retention, enhanced 24-h object recognition performance of non-habituated rats (C) but not of habituated rats (D). *P < 0.05 vs. vehicle. Adapted from (Campolongo *et al*, 2013).

Given the close relationship between the cannabinoid system and HPA axis activity (Armario, 2010; Atsak *et al*, 2012a; Barna *et al*, 2004; Hill *et al*, 2010a; Hill *et al*, 2010b), we proceeded to explore the possibility that the conditionally divergent effects of systemic WIN on object recognition memory could be related to differential effects of WIN on training-induced glucocorticoid levels. We found that WIN elevated plasma CORT levels in non-habituated rats, but decreased CORT levels in habituated rats. Most importantly, as shown in Figure 2, we demonstrated that adrenocortical suppression with the CORT-synthesis inhibitor metyrapone altered the effects of posttraining WIN administration on non-habituated rats' short- and long-term recognition memory in such a way that their cognitive performance became similar to that seen in habituated animals (Campolongo *et al*, 2013). Thus, our findings suggest that differential effects of cannabinoid receptor agonism on memory may be related to the ability of cannabinoids to interact with the HPA axis, depending on the stress state of the animal in relation to the aversiveness of the environmental conditions. A WIN-induced increase in CORT levels likely affected short-term memory performance (1-h test) in the HA condition by influencing retrieval (Atsak *et al*, 2012a). In the long-term memory experiment (24-h test), however, increased CORT levels could only have influenced consolidation. Since habituation attenuates training-induced surges in noradrenergic activity, glucocorticoids might not modulate recognition memory in habituated rats due to their relatively low levels of norepinephrine (Roozendaal *et al*, 2006b). Together, these aforementioned findings provide strong support for the view that cannabinoid-mediated regulation of glucocorticoid secretion may play an important role in determining the pattern of cannabinoid effects on memory. Although the administration of direct cannabinoid receptor agonists mimics cannabis consumption, the use of indirect agonists or antagonists can provide more focal specificity and may be particularly useful for elucidating the physiological role of the endogenous "on-demand" endocannabinoid system. Indeed, de Oliveira Alvares and co-workers (2010) reported that the hippocampal endocannabinoid system is recruited to enhance memory consolidation of contextual fear conditioning only under HA conditions. Specifically, they found that blockade of cannabinoid receptors induced by immediate posttraining infusion of the cannabinoid receptor antagonist AM251 into the dorsal hippocampus impaired 24-h memory retention of conditioning with a 0.7-mA footshock, but did not affect memory when a less aversive 0.3 mA footshock was used (de Oliveira Alvares *et al*, 2010). Hence, the hippocampal

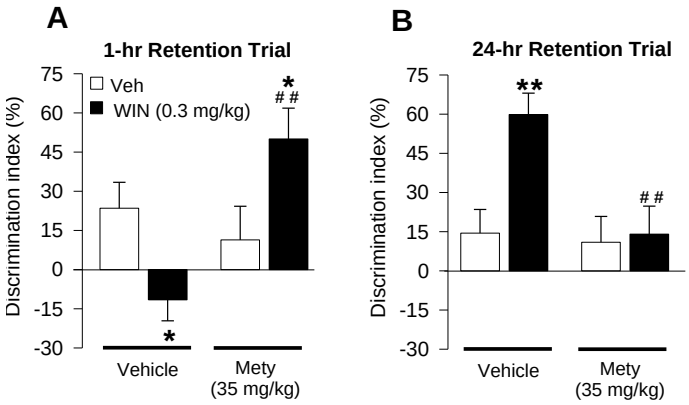

Figure 2. Effects of the CB receptor agonist WIN on short- and long-term retention of object recognition in rats trained under HA conditions and pretreated with the CORT synthesis inhibitor metyrapone (Mety). Metyrapone (35 mg/kg, i.p.) administered to non-habituated rats 40 min before training reverted the impairing effect of posttraining WIN (0.3 mg/kg, i.p.) on 1-h retention performance (A) and the enhancing effect of WIN (0.3 mg/kg, i.p.) on 24-h retention performance (B) in such a way that their performances became similar to that seen in habituated animals (compare panel C to panel A). Data are expressed as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the corresponding vehicle group; $^{\#\#}P < 0.01$ vs. WIN alone group. Adapted from (Campolongo *et al*, 2013).

endocannabinoid system may modulate memory consolidation only when there is some threshold level of aversiveness involved. Intriguingly however, Jacob and co-workers (2012) reported that CB1 knockout mice required a higher intensity footshock than wild-type mice to exhibit enhanced fear conditioning memory and generalized contextual fear (Jacob *et al,* 2012). However, with mice lacking CB1 receptors, it is not possible to discriminate which memory phase has been affected. Furthermore when a receptor is genetically deleted, other compensatory mechanisms may occur (Fraser and Wahlestedt, 1997; Giros *et al*, 1996).

Interestingly, clinical research has shown that the nature of the social setting in which a drug is taken has a direct influence on the probability of particular drug effects occurring (Porto and Masur, 1984; Zinberg, 1984). Researchers have also found that past experiences dictate the content and structure of future drug-taking experiences (Smith, 1978) and that cannabinoid drugs modulate memory for emotionally arousing experiences preferentially, without modulating memory for mundane experiences (Ballard *et al*, 2012).

Together, the preclinical and clinical findings reviewed above indicate that some degree of training-associated arousal or stress is required for glucocorticoids to affect memory consolidation and that endocannabinoids may

act as a buffer system in this regard. This interaction between stress hormones and the cannabinoid system provides support for the notion that differential sensitivity to cannabinoids may be related to the level of activation of stress pathways (Carvalho and Van Bockstaele, 2012; Oropeza *et al*, 2005; Page *et al*, 2007; Patel and Hillard, 2003). Taken together, these findings provide evidence in support of the view that the endocannabinoid system may play a key role in mediating the effects of arousal and stress on memory.

Out-of-context stress shapes cannabinoid modulation of memory

Intra-cerebroventricular administration of a CB1 receptor antagonist has been demonstrated to activate the HPA axis (Manzanares *et al*, 1999), thus indicating a central site of action despite the presence of CB1 receptors in the pituitary and adrenal glands (Cota *et al*, 2007; Ziegler *et al*, 2010). Conversely, both stress and glucocorticoids alter endocannabinoid levels in healthy volunteers subjected to stressful conditions (Feuerecker *et al*, 2012), in patients with stress-related disorders (Hauer *et al*, 2013; Hauer *et al*, 2012; Neumeister *et al*, 2013), and in limbic regions of CORT-injected rats (Hill *et al*, 2010b). This glucocorticoid-cannabinoid interaction may serve to maintain homeostatic balance. Collectively, these and other data (Hill and Tasker, 2012)—together with the broad expression of cannabinoid receptors in cortico-limbic and hypothalamic circuitry where they seem to dampen HPA axis activation (Gonzalez *et al*, 2004; Hill *et al*, 2009b; Manzanares *et al*, 1999; Newsom *et al*, 2012; Patel *et al*, 2004; Steiner *et al*, 2008a)—suggest that the endocannabinoid system could play a critical role in mediating response to stress as well as the effects of stress on memory processes.

Few studies have investigated the effects of out-of-context and non-pharmacologically induced stress and its interaction with the endocannabinoid system in modulating cognitive functions. Ganon-Elazar and Akirav (2009) have shown that infusion of the cannabinoid receptor agonist WIN into the BLA inhibits the increase in plasma CORT in rats exposed to an elevated platform stress for 30 min. Pretraining intra-BLA infusion of WIN did not induce any effect by itself, but prevented the enhancing effects of elevated platform stress on inhibitory avoidance memory and prevented the impairment of extinction induced by pre-extinction stress exposure (Ganon-Elazar *et al*, 2009). Likewise, in a food-reward-reduction straight-alley maze task, intra-BLA WIN (5 μg/side) infusions had no effect on memory consolidation alone, but blocked the memory enhancing effects of stress exposure (i.e. elevated platform for 30 min)(Ramot and Akirav, 2012). These findings appear, at least superficially, to be at odds with our observation that intra-BLA infusions of WIN (50 ng/side) immediately after inhibitory avoidance training enhanced memory consolidation (Campolongo *et al*, 2009b). These differential outcomes could be due to the different doses used or differences in the nature of the behavioral tasks employed. The inhibitory

avoidance task is an aversive, fear-motivated task, whereas the alley-maze task has a strong reward component and the reduction of a reward has been associated with a state of frustration (Spence, 1956). Interestingly, de Oliveira Alvares et al. (2010) showed that intra-hippocampal infusions of the cannabinoid receptor antagonist AM251 reverted the stress-induced facilitatory effect on memory consolidation. In this experiment, rats were trained in a contextual fear conditioning paradigm (0.3-mA footshock intensity) and tested 24 h later. AM251 had no effect *per se* on memory consolidation, but yielded a reversion to the memory enhancing effects of a preconditioning stressor (two 0.1-mA footshocks in a different context) (de Oliveira Alvares *et al*, 2010). Although it is possible that preconditioning stress might have influenced task acquisition, this finding provides further evidence that stress hormones interact with endocannabinoids in enhancing memory consolidation of aversive experiences. In light of the findings of these still limited and sometimes seemingly contradictory studies, it is becoming increasingly evident how the endocannabinoid system, by modulating stress responses (and *vice versa*), could affect memory functions differently depending on the aversiveness of the experimental conditions.

The endocannabinoid system as an emotional buffer: a possible explanation for variable cannabinoid effects on memory

It is difficult to pinpoint the exact role exerted by cannabinoid compounds on memory function given that they can induce biphasic behavioral effects that may arise from different factors and may alter sensorimotor and motivational processes (Economidou *et al*, 2007; Solinas *et al*, 2005; Steiner *et al*, 1999; Zimmer *et al*, 1999). However, growing evidence suggests that apparently conflicting findings with cannabinoid manipulations across studies may be due, perhaps in large part, to variations in the stressfulness of experimental conditions. This evidence has led us to hypothesize that the interaction of cannabinoids with stress hormones is of crucial importance in determining their modulatory effects on memory processes.

Stress effects on both consolidation and retrieval of emotionally arousing experiences require concurrent glucocorticoid and noradrenergic activity (McGaugh *et al*, 2002; Roozendaal, 2002). Stress hormone effects on memory typically follow an inverted-U shaped (rather than linear) dose-response relationship, in which modulatory effects are seen most prominently with doses in the mid-range of the inverted-U curve (Mendl, 1999; Yerkes, 1908). Although this stress-memory relationship seems not to apply to all cases with respect to several factors (i.e. stress duration, intensity, and timing in relation to memory phase) (Sandi and Pinelo-Nava, 2007), it is plausible that, depending on stress hormone levels, the subsequent interplay between endocannabinoids and glucocorticoids

and/or norepinephrine could produce opposing effects of cannabinoids on memory performance.

Cannabinoid administration can both activate and inhibit the HPA axis (Cota *et al*, 2007; Ganon-Elazar *et al*, 2009); and systemically administered CORT can produce rapid elevation of endocannabinoid levels in the amygdala (Hill *et al*, 2010a). Noradrenergic signaling within the BLA plays an important role in the modulation of memory for emotionally arousing experiences, a process highly dependent on the integrity of the BLA (McGaugh, 2000; Quirarte *et al*, 1997; Roozendaal, 2002; Roozendaal *et al*, 2004; Roozendaal *et al*, 2006a). The BLA appears to orchestrate the use of various memory systems during periods of emotional arousal, rather than serving as a substrate of long-term memory storage (Packard and Wingard, 2004). There are multiple ways in which the BLA may modulate memory processes. First, aversive tasks can augment autonomic and humoral stress responses and activate the amygdala (Roozendaal, 2002; Roozendaal *et al*, 1992). These augmented stress responses may in turn interact differentially with cannabinoids and affect cognitive functions in other brain regions such as the hippocampus and PFC. Second, the BLA can modulate cognitive functions through direct or indirect neural connections to other limbic structures. The BLA has efferents projecting to the medial PFC, nucleus accumbens, and hippocampus (Krettek and Price, 1978; Pape *et al*, 2010). Most of the amygdalo-hippocampal projections reach the ventral hippocampus, which appears to have limited involvement in learning and memory (Moser *et al*, 1993). Thus, the BLA may influence dorsal hippocampal memory processes indirectly via projections through the nucleus accumbens and entorhinal cortex. Therefore, cannabinoids could influence memory processes by modulating BLA activity and, thus, BLA efferents to other brain regions. Within the BLA, CB1 receptors are expressed abundantly by GABAergic interneurons (Katona *et al*, 2001) and activation of CB1 receptors has consistently been shown to suppress GABA release (Katona *et al*, 2001; Katona *et al*, 1999; Ohno-Shosaku *et al*, 2001) via rapid inhibition of calcium entry into the terminals (Hoffman and Lupica, 2000; Wilson and Nicoll, 2001). Moreover, the amygdalar GABAergic system modulates memory storage (McGaugh, 2000) and inhibition of GABAergic activity within the BLA enhances memory consolidation by increasing the release of norepinephrine (Hatfield *et al*, 1999). We demonstrated recently that endocannabinoids in the BLA enhance memory consolidation for an emotionally salient event by interacting with glucocorticoids (Campolongo *et al*, 2009b). Indeed, we found that intra-BLA administration of the CB1 receptor antagonist AM251 blocked the ability of systemically administered CORT to facilitate memory consolidation of inhibitory avoidance training (Campolongo *et al*, 2009b). These findings showed, for the first time *in vivo*, that glucocorticoids recruit endocannabinoid signaling in the BLA while modulating aversive memory consolidation (Hill and McEwen,

2009a; Hill *et al*, 2010c). Moreover, de Oliveira Alvares and co-workers demonstrated that intra-hippocampal AM251 infusion impaired memory in rats that had received a synthetic glucocorticoid (dexamethasone) injection immediately after, but not 30 min before training. Their study demonstrated that, in the context of modulation of aversive memory consolidation, hippocampal endocannabinoid transmission is activated in a time-dependent manner and interacts with glucocorticoids (de Oliveira Alvares *et al*, 2010).

An interaction between glucocorticoids and endocannabinoids in modulating memory retrieval has also been examined recently. Infusion of the cannabinoid receptor antagonist AM251 into the dorsal hippocampus blocked the retrieval impairing effects of systemic CORT, which were dependent upon elevation of hippocampal 2-AG levels (Atsak *et al*, 2012a). Moreover, the β-adrenoceptor antagonist propranolol blocked the impairing effect of WIN on memory retrieval and, conversely, infusion of the CB1 receptor antagonist AM251 into hippocampus together with an impairing dose of norepinephrine failed to abolish the impairing effect of norepinephrine on memory retrieval (Atsak *et al*, 2012a). Collectively, these findings indicate that endocannabinoids interact with glucocorticoids and may modulate memory functions differentially depending on the activation state of the noradrenergic system.

In view of this evidence, we have proposed a model in which CORT binds to membrane bound receptors in the BLA that activate a G-protein signaling cascade to stimulate the synthesis of endocannabinoids. Once in the synaptic cleft, endocannabinoids may inhibit GABA release from presynaptic terminals, which in turn may lead to disinhibition of norepinephrine release and increased noradrenergic activation of postsynaptic β-adrenoceptors, enhancing the consolidation of emotionally aversive memories (Fig. 3) (Atsak *et al*, 2012b; Campolongo *et al*, 2009b; Hill and McEwen, 2009). There are several characteristics of the endocannabinoid system that should be considered and might be involved in the dual cannabinoid effects on memory. First, endocannabinoids are synthesized and released on-demand and, as a result, they are released only in those brain regions where and when there is active endocannabinoid signaling. Brain endocannabinoid responses, and the relative activation of the endocannabinoid system in discrete brain areas, may vary depending on the nature and intensity of environmental stimuli. Interestingly, pharmacological manipulations with indirect cannabinoid receptor agonists or antagonists increase and block, respectively, the endocannabinoid response only in those brain areas where signaling was concurrently active. Conversely, direct agonists bind all cannabinoid receptors in the brain and the periphery, regardless of their involvement in a particular process.

Second, CB1 receptors are widely expressed in brain regions that play key roles in responding to stressful stimuli. It is possible that CB1 receptors produce

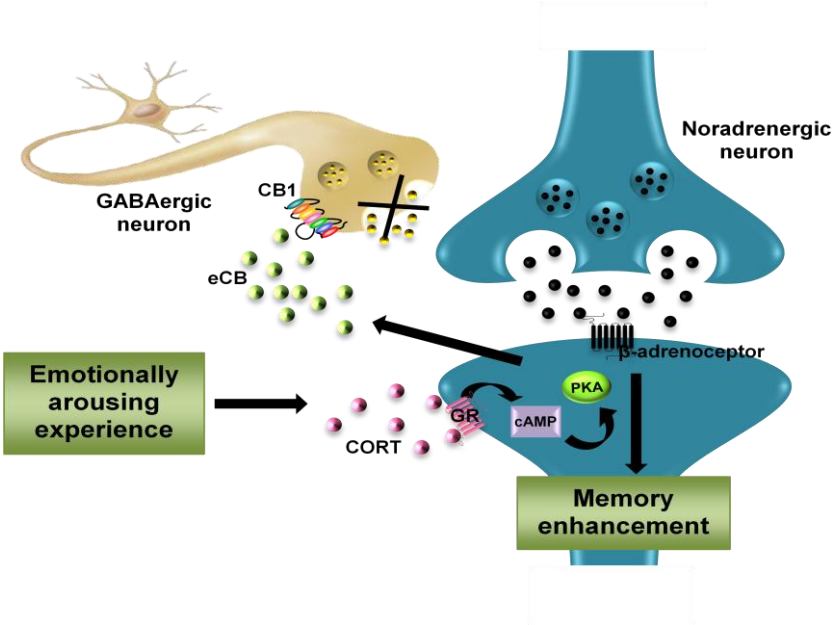

Figure 3. Model of endocannabinoid role in the modulation of memory consolidation within the BLA. Stress hormones (i.e. CORT and epinephrine) are released into the bloodstream during training. CORT binds metabotropic GRs within the BLA, activating the G_s–cAMP/PKA pathway to induce endocannabinoid (eCB) synthesis. Endocannabinoids are released into the synaptic cleft where they bind CB1 receptors on GABAergic terminals, thereby inhibiting GABA release. Suppression of GABAergic transmission results in the disinhibition of noradrenergic neurons and increases noradrenergic activation of postsynaptic β-adrenoreceptors, enhancing the consolidation of emotionally aversive memories. Adapted from (Hill and McEwen, 2009).

opposite behavioral effects, depending on their anatomical location. Since CB1 receptors are expressed presynaptically, they can suppress the release of neurotransmitters such as GABA and glutamate (Azad *et al*, 2008; Kano *et al*, 2009; Marsicano *et al*, 1999; Monory *et al*, 2006), which often act in opposition to each other in the control of neurophysiological processes related to memory and emotional responses (Chevaleyre *et al*, 2006; Metna-Laurent *et al*, 2012; Millan, 2003; Myhrer, 2003). Furthermore, the densities of the molecular endocannabinoid system components differ between synapse types in general (i.e. glutamatergic vs. GABAergic) and among the great variety of individual synapses expressing cannabinoid receptors (Katona and Freund, 2012). For instance, the highest density of CB1 receptors is found on cholecystokinin-positive GABAergic synapses in the hippocampus (Katona *et al*, 2000; Katona *et al*, 1999), and much lower levels of CB1 receptors are found on glutamatergic synapses (Katona *et al*, 2006; Kawamura *et al*, 2006). Hence, cannabinoid influences on

behavior may differ qualitatively depending on the synapses activated and neuronal circuits recruited in a particular situation. CB1 receptors might shape the environmental impact on memory functions by balancing inhibitory and excitatory neuronal activity. The functional interaction of the endocannabinoid system with these inhibitory or excitatory neurotransmitters may consequently be particularly relevant for the dual effects of cannabinoids in learning and memory processes. A small change in the environment might recruit new neurons in a different circuit, changing the location and the neurochemical nature of the cannabinoid-modulated synapses that were activated. This unique characteristic makes the endocannabinoid system well suited to serve as a buffer system to balance emotional reactivity and ensure an appropriate stress response.

Furthermore, the capacity of endocannabinoids to activate receptors other than CB1 should be considered. CB2 receptors were also proposed to be relevant for emotional responses (Onaivi *et al*, 2006). Moreover, endocannabinoids also activate the peroxisome proliferator-activated nuclear receptor (O'Sullivan, 2007), which modulates both aversive memory consolidation (Campolongo *et al*, 2009a) and acquisition (Mazzola *et al*, 2009), and the transient receptor potential vanilloid type 1 (Starowicz *et al*, 2007) which has been shown to mediate opposing effects on emotional responses with respect to CB1 (Maione *et al*, 2006).

Conclusions

The findings reviewed here shed light on the divergent effects of cannabinoids on memory processes reported in the literature, indicating that environmental events characterized by different levels of stress can shape responses to the cognitive effects of cannabinoids. Given its modulatory role, we propose that the endocannabinoid system may moderate environmental impacts on emotional memory and attenuate excessive behavioral responses to stress. As a key modulator of environmental and stress influences on memory, the endocannabinoid system should be explored as a possible therapeutic target for neuropsychiatric illness involving memory dysfunction, such as post-traumatic stress disorder.

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CONCLUSION

Conclusion

This thesis explored the role of the endocannabinoid system in the modulation of cognitive function and its interaction with stress and emotional arousal in determining modulatory effects on memory processes.

First, we investigated the neurobiological mechanisms underlying memory consolidation for emotionally arousing experiences and demonstrated that the endocannabinoid system within the basolateral complex of the amygdala plays an important role in regulating aversive memory formation.

We, then, examined the interaction between the endocannabinoid system and stress hormones in the modulation of emotional memory processes, demonstrating that, depending on the availability of stress hormones, the subsequent interplay between endocannabinoids and glucocorticoids and/or norepinephrine results in opposing effects on memory processes. We pointed out that the endocannabinoid system shapes stress effects on memory to prepare the organism for similar challenges in the future.

The concept of emotion regulation is considered critical to healthy emotional functioning and is disrupted in a variety of different types of psychopathology. Intensely emotional events or exposure to stressful experiences can create traumatic memories and even result in the development of mood and anxiety disorders, including post-traumatic stress disorder and major depressive illness. It is of considerable importance to better understand the brain mechanisms related to cognitive processes to shed light on the neural underpinnings of several cognitive disorders that are not yet well understood and current therapeutic tools are not always successful to treat such disorders. Therefore, to study the role of the endocannabinoid system in cognitive processes would help to provide new therapeutic approaches for the treatment of stress-induced psychopathological alterations associated with cognitive disturbances.

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PUBLICATIONS (PEER REVIEWED JOURNALS)

Morena M. et al., *Endocannabinoids enhance memory consolidation of emotionally arousing experiences: key role of the basolateral complex of the amygdala.* In Preparation.

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Morena M., Trezza V., Scaccianoce S., Peloso A., Cuomo V., Roozendaal B., Campolongo P. Training-associated emotional arousal differentially modulates cannabinoid effects on recognition memory and adrenocortical activity in rats – "Frontiers in Stress and Cognition: From Molecules to Behavior" - September 23-26, 2012, Ascona, Switzerland

Morena M., Campolongo P., Cuomo V. Interaction between cannabinoids and glucocorticoids in the modulation of recognition memory in rats - 16° Seminario SIF Dottorandi e Assegnisti di Ricerca - September 16-19, 2012, Rimini, Italy

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Morena M., Trezza V., Scaccianoce S., Pasquale A., Cuomo V., Schelling G., Roozendaal B., Campolongo P. Cannabinoid modulation of object recognition memory in rats: involvement of the glucocorticoid system- 22th Annual symposium of the International Cannabinoid Research Society - July 22-27, 2012, Freiburg, Germany

Morena M., Hauer D., Ratano P., Scaccianoce S., Trezza V., Pecci C., Atsak P., Cuomo V., Roozendaal B., Schelling G., Campolongo P. The endocannabinoid system and the regulation of memory consolidation for emotionally arousing experiences - 8th FENS Forum of Neuroscience - July 14-18, 2012, Barcelona, Spain

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Morena M., Trezza V., Ratano P., Pecci C., Palmery M., Campolongo P., Cuomo V. Inhibition of fatty acid amide hydrolase in the rat limbic system enhances consolidation of memory for emotionally arousing events – 35°congresso nazionale della società italiana di farmacologia “Il farmaco: dalla ricerca alla salute dell’uomo” - September 14-17, 2011, Bologna, Italy

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